Intersex-like (*IXL*) Is a Cell Survival Regulator in Pancreatic Cancer with 19q13 Amplification

Riina Kuuselo,[1] Kimmo Savinainen,[1] David O. Azorsa,[2] Gargi D. Basu,[2] Ritva Karhu,[1] Sukru Tuzmen,[2] Spyro Mousses,[2] and Anne Kallioniemi[1]

[1]Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, Tampere, Finland and [2]Pharmaceutical Genomics Division, The Translational Genomics Research Institute, Scottsdale, Arizona

Abstract

Pancreatic cancer is a highly aggressive disease characterized by poor prognosis and vast genetic instability. Recent microarray-based, genome-wide surveys have identified multiple recurrent copy number aberrations in pancreatic cancer; however, the target genes are, for the most part, unknown. Here, we characterized the 19q13 amplicon in pancreatic cancer to identify putative new drug targets. Copy number increases at 19q13 were quantitated in 16 pancreatic cancer cell lines and 31 primary tumors by fluorescence *in situ* hybridization. Cell line copy number data delineated a 1.1 Mb amplicon, the presence of which was also validated in 10% of primary pancreatic tumors. Comprehensive expression analysis by quantitative real-time reverse transcription-PCR indicated that seven transcripts within this region had consistently elevated expression levels in the amplified versus nonamplified cell lines. High-throughput loss-of-function screen by RNA interference was applied across the amplicon to identify genes whose down-regulation affected cell viability. This screen revealed five genes whose down-regulation led to significantly decreased cell viability in the amplified PANC-1 cells but not in the nonamplified MiaPaca-2 cells, suggesting the presence of multiple biologically interesting genes in this region. Of these, the transcriptional regulator intersex-like (*IXL*) was consistently overexpressed in amplified cells and had the most dramatic effect on cell viability. *IXL* silencing also resulted in G_0-G_1 cell cycle arrest and increased apoptosis in PANC-1 cells. These findings implicate *IXL* as a novel amplification target gene in pancreatic cancer and suggest that *IXL* is required for cancer cell survival in 19q13-amplified tumors. [Cancer Res 2007;67(5):1943–9]

Introduction

Pancreatic adenocarcinoma is a highly aggressive malignancy with extremely poor prognosis. In the United States, pancreatic cancer is the fifth leading cause of cancer death, accounting for ~30,000 deaths per year (1). Pancreatic cancer is characterized by rapid progression, invasiveness, and profound resistance to treatment (2). Apart from surgery, there is practically no effective therapy; typically, the disease is diagnosed at an advanced stage when surgical resection is no longer possible. Consequently, the 5-year survival rate for pancreatic cancer is <5% and the median survival is <6 months (2, 3). Even for patients who undergo potentially curative resection, the 5-year survival rate is only ~20% (2).

Aneuploidy and increased genetic instability manifesting as complex genetic aberrations, such as losses, gains, and amplifications, are common features of pancreatic cancer (4, 5). These genetic alterations are likely to conceal genes involved in disease pathogenesis, and uncovering such genes might thus provide targets for the development of new diagnostic and therapeutic tools. In particular, gene amplification is a common mechanism for activating oncogenes, and other growth-promoting genes in cancer and amplification of target genes, such as *ERBB2* in breast cancer and *MYCN* in neuroblastomas, have been shown to have clinical significance as diagnostic and prognostic markers as well as therapeutic targets (6). We recently did a microarray-based copy number analysis in pancreatic cancer cell lines and identified an ~2.9 Mb amplicon at 19q13 (7). This result has since been confirmed in multiple subsequent studies (8–12) and the same amplicon has also been observed in other tumor types, including ovarian, breast, cervical, gastric, and small-cell lung cancer (13–20). Unfortunately, the microarray used in our previous study did not provide a complete coverage of the 19q13 region and thus did not allow direct elucidation of putative target genes. Here, we present results from the comprehensive evaluation of the 19q13 amplicon in pancreatic cancer, including detailed copy number and expression analyses as well as high-throughput loss-of-function screen using the RNA interference (RNAi) technology.

Materials and Methods

Cell lines and tissue samples. Thirteen pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, HPAF-II, Hs 700T, Hs 766T, MiaPaCa-2, PANC-1, SU.86.86, and SW1990, were obtained from the American Type Culture Collection (Manassas, VA). Three additional cell lines, DAN-G, HUP-T3, and HUP-T4, were acquired from the German Collection of Microorganisms and Cell Cultures (Brunswick, Germany). The cell lines were cultured under recommended conditions. Commercially available pancreatic cancer tissue microarray (AccuMaxTM Arrays) was obtained from Petagen Incorporation (Seoul, Korea). The tissue microarray contained four nonneoplastic pancreatic tissue specimens and 33 pancreatic cancer cases. Detailed clinicopathologic information on the tumor specimens is shown in Supplementary Table S1.

Genomic clones. Public genome databases (National Center for Biotechnology Information[3] and University of California Santa Cruz Genome Bioinformatics[4]) were used to select 15 bacterial artificial chromosome (BAC) clones evenly distributed over the 2.9 Mb amplicon at 19q13. These BAC clones were obtained from Invitrogen (Carlsbad, CA).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Anne Kallioniemi, Institute of Medical Technology, Biokatu 6, 33014 University of Tampere, Tampere, Finland. Phone: 358-3-3551-8833; Fax: 358-3-3117-4168; E-mail: anne.kallioniemi@uta.fi.

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[3] http://www.ncbi.nlm.nih.gov/

[4] http://www.genome.ucsc.edu/

In addition, a BAC clone (RP11-345J21, a kind gift from Mariano Rocchi, University of Bari, Bari, Italy) adjacent to the chromosome 19 centromere on 19q was used as a control. The list of all BAC clones is shown in Supplementary Table S2.

Fluorescence *in situ* hybridization. BAC clone DNA was labeled with SpectrumOrange-dUTP (Vysis, Downers Grove, IL) using random priming. Chromosome 19 reference probe (RP11-345J21) was labeled with fluorescein-12-dUTP (Perkin-Elmer, Boston, MA). Dual-color interphase fluorescence *in situ* hybridization (FISH) to pancreatic cancer cell lines was done as described (21). Hybridization signals were evaluated using Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan). Forty intact nuclei were scored for each probe, and relative copy numbers were calculated as ratios of mean absolute copy number of the test probe versus reference probe. FISH on tissue microarray was carried out as described (22). Three adjacent, partly overlapping BAC clones (RP11-67A5, RP11-256O9, and CTC-488F21) were combined to increase signal intensity and hybridization efficiency. Control experiments on normal lymphocytes verified that this probe combination gave a single hybridization signal. The RP11-345J21 probe was again used as a reference. Hybridization signals were scored and evaluated as described above.

Quantitative real-time reverse transcription-PCR. Gene expression analyses were done using either the Light Cycler equipment (Roche, Mannheim, Germany) or the ABI 7900HT Fast Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA was isolated from cell lines using RNeasy Mini total RNA extraction kit (Qiagen, Valencia, CA) and reverse transcribed into first-strand cDNA using Superscript III reverse transcriptase (Invitrogen) and random hexamers or iScript cDNA synthesis kit (Bio-Rad, Inc., Hercules, CA). Normal human pancreatic RNA was obtained from Ambion (Cambridgeshire, United Kingdom). For the Light Cycler analyses, primers and probe sets were obtained from TIB MolBiol (Berlin, Germany), and Light Cycler software (Roche) was used for data analysis as described (23). Expression levels of the target genes were normalized against a housekeeping gene *TBP* (TATA box binding protein; ref. 23). For the ABI system, TaqMan Gene Expression Assays were obtained from Applied Biosystems and ABI 7900HT software (Applied Biosystems) was used for data analyses. Expression levels of the target genes were normalized against an endogenous reference gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). Primer and probe sequences are shown in Supplementary Table S3.

Loss-of-function RNAi screen. Loss of function screening was done by high-throughput RNAi using a focused small interfering RNA (siRNA) library targeting all 19 known genes within the amplicon that were expressed in PANC-1 cells (Supplementary Table S4). Two genes, *SAMD4B* and *EID2B*, were hypothetical proteins at the time of the experiment and were thus not included. Four siRNAs were designed for each gene using previously described criteria (24, 25) and were obtained from Qiagen. A nonsilencing siRNA (Qiagen) was used as a negative control. High-throughput RNAi was done using siRNA reverse transfection of cells. Briefly, siRNAs were printed in quadruplicate wells of a 384-well Costar microtiter plate (Fisher Scientific, Hampton, NH). Diluted Oligofectamine (Invitrogen) was added to the wells to allow for the complexing of siRNA and transfection reagent. After a 30-min incubation period, cell suspensions of either MiaPaca-2 or PANC-1 cells were added to give a final concentration of 1,000 per well. Cells were grown at 37°C with 5% CO_2 for 96 h. Total cell number was analyzed using Cell Titer Blue (Promega, Madison WI), and the plate was read at excitation 544 nm/emission 560 nm using a EnVision plate reader (Perkin-Elmer, Wellesley, MA). Reduced cell viability to 50% or less compared with nonsilencing siRNA-treated cells was considered a significant change.

Cell cycle and apoptosis analyses. Oligofectamine reagent (Invitrogen) was used to transfect siRNAs in a final concentration of 100 nmol/L into PANC-1 cells according to the manufacturer's protocol. *IXL* 144 siRNA was used to determine the effect of inhibition of IXL expression; luciferase control siRNA, which targets the firefly *luciferase* gene (Genbank accession no. M15077), was used as a control (Supplementary Table S4). Experiments were done in triplicates using 24-well plates. After a 48-h transfection, cells were collected for cell cycle and apoptosis analyses as well as for parallel mRNA expression analyses (see above) to verify that efficient silencing was obtained. For the cell cycle analysis, trypsinized cells were centrifuged and suspended to 500 μL hypotonic staining buffer (0.1 mg/mL sodium citrate tribasic dehydrate, 0.03% Triton X-100, 50 μg/mL propidium iodide, 2 μg/mL RNase A) and the amount of propidium iodide incorporated was determined using flow cytometry (Coulter EPICS XL-MCL, Beckman Coulter, Inc., Fullerton, CA). Cell cycle distribution was analyzed using the Cylchred program.[5] Annexin V FITC Apoptosis Detection Kit (Calbiochem) was used to detect apoptotic cells by flow cytometry (Beckman Coulter, San Diego, CA).

Results

We first did a systematic characterization of the extent of the 19q13 amplicon in a panel of 16 pancreatic cancer cell lines. To this end, FISH, using 15 BAC clones evenly distributed across the 2.9 Mb region (Supplementary Table S1), was applied. Greater than 2-fold copy number increases were observed in 3 of 16 (19%) cell lines, PANC-1, Su.86.86, and HPAC (Fig. 1). The PANC-1 cell line harbored massive amplification (up to 20-fold) that on metaphase chromosomes manifested as homogeneously staining region–like structures (Fig. 1*E*), whereas lower-level copy number increases (up to 5.3- and 2.6-fold, respectively) were observed in SU.86.86 and HPAC cells (Fig. 2). The relative copy numbers of PANC-1 cells varied considerably across the 2.9 Mb region (Fig. 1*A–D*), whereas SU.86.86 and HPAC cells showed essentially uniform copy number levels across the entire amplicon. Based on the PANC-1 copy number profile, we were able to delineate a 1.1 Mb amplicon core defined by six partly overlapping BAC clones from RP11-67A5 to CTC-425O23 (Fig. 2). Furthermore, three BAC clones within this 1.1 Mb amplicon core, RP11-67A5, RP11-256O9, and CTC-488F21, displayed 14- to 20-fold copy number increase in PANC-1 cells (Fig. 2), thus defining an 660 kb subregion of extremely high level amplification.

We also examined the presence of this amplicon in primary tumors using FISH to a tissue microarray containing 33 pancreatic cancer specimens. High-level amplification (relative copy number $\geq$5) was observed in 3 of 31 tumors (9.7%) with successful hybridizations (Fig. 3). All three tumors with amplification were moderately to poorly differentiated (grades 2–3) ductal adenocarcinomas and showed lymph node metastases (Supplementary Table S2). Two of the tumor samples also had extensions to peripancreatic soft tissue with perineural invasion. The metastasis status of the third tumor was not available.

To explore the consequences of amplification on gene expression, we first used public genome databases[6] to retrieve all transcribed sequences within the core 1.1 Mb amplicon. A total of 39 transcripts, including 27 known genes and 12 hypothetical or predicted proteins, were identified (Table 1). Sixteen of these were excluded as possible candidate genes because they represented (*a*) obvious pseudogenes, (*b*) predicted transcripts with no mRNA and expressed sequence tag evidence in public databases, or (*c*) transcripts with no or very low level expression in PANC-1 (Table 1). The last criterion was based on the expectation that any putative amplification target gene should be highly expressed in PANC-1 cells that show high-level amplification. The expression levels of the remaining 23 transcripts were then assessed in all 16 pancreatic cancer cell lines using quantitative real-time RT-PCR. Interestingly, 22 of the 23 genes were highly overexpressed in

[5] http://www.cardiff.ac.uk/medicine/haematology/cytonetuk/documents/software.htm

[6] http://www.genome.ucsc.edu and http://www.ncbi.nlm.nih.gov

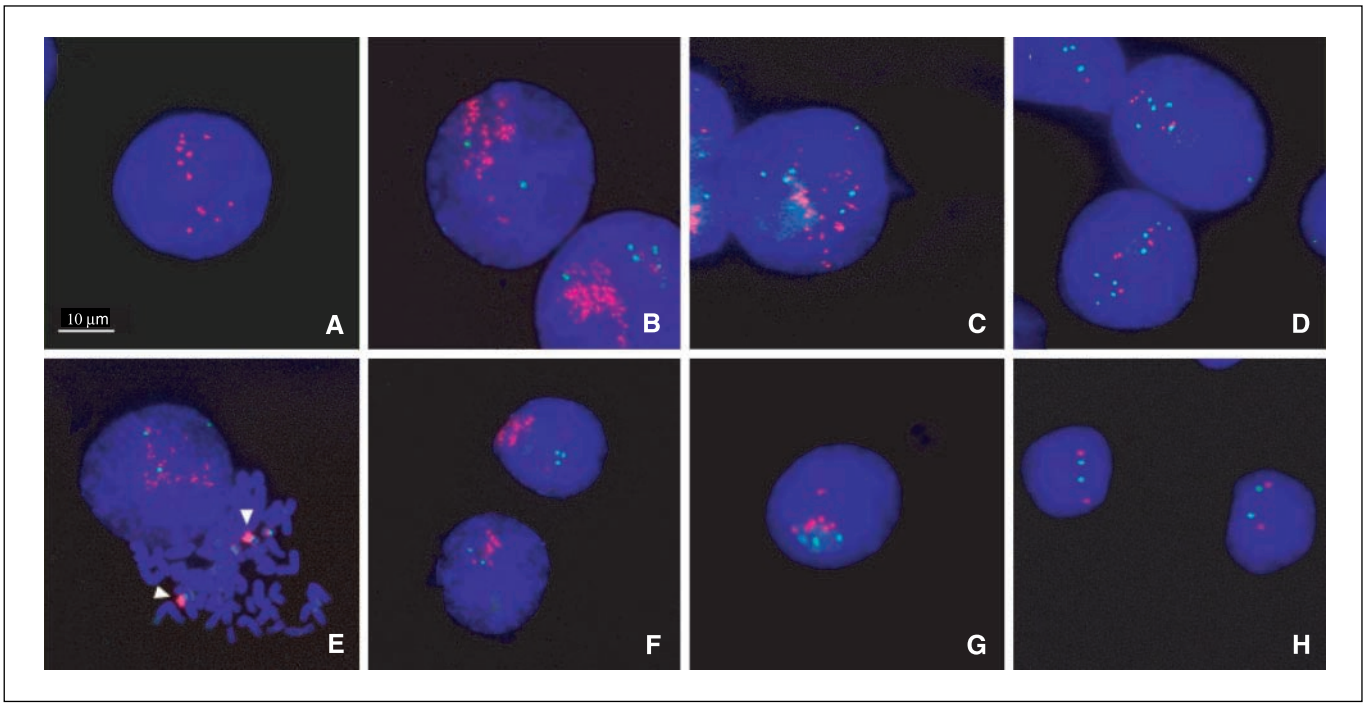

Figure 1. Copy number increases at 19q13 in pancreatic cancer cell lines. *A* to *D,* FISH signals in PANC-1 cells across the amplicon using BAC clones (*red signals*) CTC-218B8 (*A*), CTC-488F21 (*B*), RP11-246P10 (*C*), and CTC-492K19 (*D*), with a chromosome 19 pericentromeric reference probe (*green signals*). *E,* amplification manifesting as homogenously staining regions on PANC-1 metaphase chromosome (*arrows*). Two apparently normal copies of chromosome 19 are seen in the same metaphase. Low-level copy number increases in SU.86.86 (*F*) and HPAC (*G*) as well as no copy number change in Capan-1 cells (*H*). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*blue*). *Bar* (in *A*), 10 μm, for all panels.

PANC-1 cells compared with the other pancreatic cancer cell lines (Fig. 4) and normal pancreas (data not shown). Only *PLD3*, located at the distal-most end of the amplicon, was not overexpressed in PANC-1. In contrast, HPAC and Su.86.86 cells showed variable expression levels from one gene to another (Fig. 4) thus allowing us to distinguish genes differentially expressed between amplified and nonamplified cell lines. Six known genes (*GMFG, SAMD4B, IXL, SUPT5H, PSMC4*, and *MAP3K10*) and one hypothetical protein (*LOC284323*) showed consistent overexpression in all three amplified cell lines (Fig. 4). Of these, *IXL* showed the most distinct differential expression pattern between the amplified and nonamplified cell line groups, with high-level expression occurring almost exclusively in the amplified cell lines. In contrast, *GMFG* and *MAP3K10* were also highly expressed in additional cell lines with no copy number increase, indicating other activating mechanisms besides amplification. Interestingly, three of the overexpressed genes, *PSMC4, MAP3K10*, and *LOC284323*, are located outside the 660 kb amplicon maximum, thus making them less likely to be the main targets of the amplification.

To identify which of the amplified targets are functionally important, we did a targeted high-throughput RNAi screen across the defined 1.1 Mb amplicon. This loss-of-function survey was applied to study the effect of silencing of 19 known genes from the amplified region. Four different siRNAs were designed for each gene (Supplementary Table S4), and their effect on cell viability was examined in PANC-1 and MiaPaCa-2 cells 96 h after transfection. Down-regulation of *IXL* resulted in statistically highly significant reduction in cell viability ($P < 0.001$) in the amplified PANC-1 cells but not in the nonamplified MiaPaCa-2 cells (Fig. 5*A*; Supplementary Table S5). This effect was observed with two independent siRNAs in three repeated experiments. Similar reduction in cell viability was detected after *LRFN1* and *PLEKHG2* silencing as well but only with a single siRNA. Finally, down-regulation of *GMFG* and *SUPT5H* also led to reduction in cell viability but to a lesser extent (Fig. 5*A*). Other genes examined did not have a statistically significant effect on cell viability.

Further functional characterization concentrated on *IXL* because it was located within the 660 kb amplicon maximum and was implicated in both the expression survey and the RNAi viability

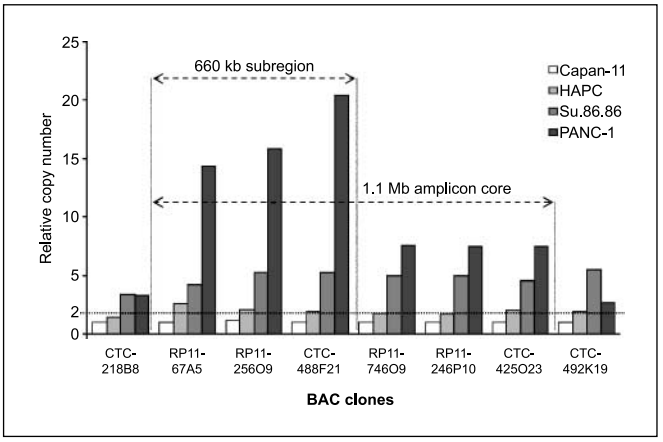

Figure 2. Amplicon mapping at 19q13 in pancreatic cancer cell lines. Relative copy number ratios for eight BAC clones around the minimal amplified region are shown for the three amplified cell lines, PANC-1, SU.86.86, and HPAC, as well as a representative nonamplified cell line, Capan-1. *Vertical dashed lines,* the 1.1 Mb core region of amplification (defined by clones RP11-67A5 and CTC-425O23) as well as the 660 kb subregion (defined by clones RP11-67A5 and CTC-488F21).

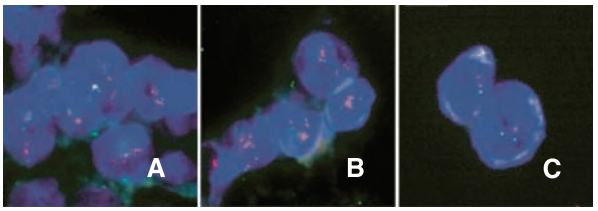

Figure 3. Copy number analysis in primary pancreatic tumors by FISH. A contig of three adjacent probes (RP11-67A5, RP11-256O9, CTC-488F21; *red signals*) was hybridized together with the chromosome 19 pericentromeric control probe (*green signals*) to a tissue microarray containing 33 pancreatic tumor samples. Examples of tumors with (*A–B*) and without (*C*) amplification.

screen. To explore the functional role of *IXL* overexpression, we transfected siRNAs targeting *IXL* into amplified PANC-1 cells and assessed the effects on cell cycle and apoptosis 48 h after transfection. A parallel mRNA expression analysis was done to verify that sufficient silencing was obtained (Fig. 5*B*). All functional experiments were done in triplicates and repeated twice. Cell cycle analysis using flow cytometry showed an increased fraction of G_0-G_1 phase cells after *IXL* siRNA (66%) transfection compared with nontransfected cells (37%) or cells transfected with a control siRNA (39%; Fig. 5*C*). Also, the percentage of apoptotic cells was increased in *IXL* siRNA–treated cells (8%) compared with control siRNA (1.6%) or untreated cells (2.5%; Fig. 5*D*). Similar phenotypic changes were not observed after *IXL* siRNA transfection to nonamplified MiaPaCa-2 cells (data not shown).

Discussion

Gross chromosomal aberrations, including losses, gains, and amplifications, are frequent in pancreatic cancer (3). Gene amplification is a common mechanism for solid tumors to upregulate the expression of genes involved in tumor progression (26, 27). Although multiple amplified regions have been documented in pancreatic carcinoma, the putative target genes activated by these aberrations are largely unknown. Identification of novel amplification target genes is extremely important because it will not only advance our knowledge on pancreatic cancer pathogenesis but it might also provide new tools for the clinical management of this highly aggressive disease.

Previously, we did a genome-wide cDNA microarray-based copy number analysis to identify localized DNA amplifications in pancreatic cancer and recognized a novel amplified region at 19q13 spanning 2.9 Mb (7). However, due to incomplete clone coverage on the microarray used, this previous study did not permit exact definition of the amplicon boundaries or direct identification of possible amplification target genes. Therefore, we now carried out a systematic evaluation of this amplified region to achieve these objectives. Three cell lines, PANC-1, SU86.86, and HPAC, harbored the 19q13 amplification, with PANC-1 cells demonstrating a massive, up to 20-fold, amplification. In addition, the copy number profile of PANC-1 allowed us to narrow down the amplicon core to 1.1 Mb, which also contained a 660 kb subregion of extremely high level amplification. Previous studies have indicated that amplification target genes are likely to be located at or near the center of the amplification maximum; that is, the region with highest copy number increase (28, 29). We thus believe this 660 kb subregion to be of particular interest in pinpointing the actual target genes of this amplicon.

Because the delineation of the amplicon core was accomplished using established pancreatic cancer cell lines, we next sought to validate the presence of the 19q13 amplicon in primary pancreatic tumors. Evaluation of a set of 31 pancreatic tumors revealed amplification in a $\sim$10% of the cases, thus confirming that this aberration is also present in actual human tumors and is not a cell culture–derived artifact. In general, oncogene amplification has been shown to be linked to advanced disease (30, 31), and, in ovarian carcinoma, 19q13 amplification has been associated with less differentiated and more aggressive tumors (14). In our series, all three tumors with amplification were moderately to poorly differentiated. All three patients had nodal metastases and two of them also had local metastases, whereas this information was missing from the third patient. Based on these tumor characteristics, the 19q13 amplification seems to be associated with advanced disease in pancreatic cancer as well. However, the number of analyzed tumors was limited; therefore, the amplification frequency as well as the possible clinicopathologic associations need to be confirmed.

A comprehensive expression analysis was subsequently done across the 1.1 Mb region to identify genes whose expression levels are elevated through 19q13 amplification. This approach was based on the well-established concept that amplification leads to increased expression of the putative target gene. Our data revealed a very distinct expression profile in PANC-1 cells demonstrating high-level expression of all but one gene throughout the entire amplicon. We hypothesize that the extremely high-level amplification in PANC-1 cells leads to complete deregulation of transcriptional control across the amplicon and thereby increased expression of all genes within this region. In contrast, SU.86.86 and HPAC cells displayed more variable expression patterns with consistent overexpression in a subset of seven genes compared with nonamplified cells, with *IXL* having the strongest association with amplification. Four of these seven genes, *GMFG, SAMD4B, IXL*, and *SUPT5H*, are located within the 660 kb amplicon maximum and were therefore considered the most likely targets.

Because the expression analysis did not explicitly pinpoint a single putative amplification target gene, we chose to perform a targeted high-throughput RNAi screen across the entire amplicon to identify functionally relevant genes. Combination of data from the expression analysis and the RNAi screen allowed us to rapidly and systematically identify genes whose expression levels were elevated through amplification, and, at the same time, whose down-regulation resulted in phenotypic changes. This strategy highlighted *IXL* as a gene that is activated by the 19q13 amplification and whose down-regulation resulted in most dramatic reduction in cell viability in amplified PANC-1 but not in nonamplified MiaPaCa-2 cells. *LRFN1* and *PLEKH2G* knockdowns also affected cell viability but not as consistently as *IXL*. However, these genes were not systematically overexpressed in all amplified cell lines. Down-regulation of *SUPT5H* and *GMFG* also depressed cell viability; however, the decrease was not as significant as for *IXL*. Based on these data, *IXL* seems to be the strongest candidate for the amplification target gene; however, we cannot exclude the involvement of some of the other genes in the region.

Further functional assays showed that *IXL* silencing resulted in increased apoptosis and G_0-G_1 arrest again in PANC-1 cells but not in MiaPaCa-2 cells, suggesting that *IXL* affects cell cycle regulatory

mechanisms that control the G_1-S transition as well as induction of apoptosis. These data implicate that *IXL* is required for cell cycle progression and cell survival in 19q13-amplified pancreatic cancer cells. *IXL* is a homologue to *Drosophila melanogaster intersex*, a transcriptional regulator involved in female somatic sex determination (32). The protein is broadly conserved during evolution (33), suggesting its importance in transcriptional regulation also in other species. Indeed, mammalian IXL has been recognized as a subunit of Mediator, a multiprotein complex that transduces regulatory signals from DNA-binding transcription factors to RNA polymerase II and thereby regulates mRNA synthesis (33, 34). The Mediator complex is required for transcriptional activation and thus controls key cellular processes. The exact function of human IXL remains elusive, although it was recently proposed to be involved in mitogen-activated protein kinase signaling pathway (33). The overexpression of IXL in pancreatic cancer may thus lead to inappropriate activation of several critical cellular processes, such as those regulating cell growth.

The *AKT2* gene has previously been suggested as the target for the 19q13 amplification (15). *AKT2* is the human homologue of the viral v-akt oncogene, which is responsible for leukemia in mice (19). Amplification of *AKT2* was originally discovered in ovarian cancer (15) but has been later observed also in other cancer types (14). Our data show that *AKT2* is indeed amplified in pancreatic cancer, although it is located at the distal-most end of the 19q13 amplicon. However, *AKT2* was not consistently overexpressed in all amplified cell lines and its down-regulation did not affect cell viability. These findings indicate that *AKT2* is clearly not the main target of the 19q13 amplification in pancreatic cancer although it cannot be ruled out that simultaneous activation of *AKT2* with other

Table 1. List of genes in the 19q13 amplicon

Gene name	Start	Stop	Description	Comment
IL29	44,478,805	44,481,152	Interleukin 29	Not expressed*
LRFN1	44,489,048	44,503,338	Leucine-rich repeat and fibronectin type III domain containing 1	
GMFG	44,510,839	44,518,460	Glia maturation factor γ	
SAMD4B	44,539,246	44,568,186	Sterile α motif domain containing 4B	
PAF1	44,568,112	44,573,519	Paf1, RNA polymerase II associated factor, homologue (*S. cerevisiae*)	
IXL	44,573,840	44,583,048	Intersex-like (*Drosophila*)	
ZFP36	44,589,327	44,591,885	Zinc finger protein 36, C3H type, homologue (mouse)	
PLEKHG2	44,595,590	44,607,994	Pleckstrin homology domain containing family G member 2	
RPS16	44,615,692	44,618,478	Ribosomal protein S16	
SUPT5H	44,628,164	44,659,150	Suppressor of Ty 5 homologue (*S. cerevisiae*)	
TIMM50	44,663,316	44,674,306	Translocase of inner mitochondrial membrane 50 homologue (yeast)	
DLL3	44,681,427	44,690,949	Delta-like 3 (*Drosophila*)	
SELV	44,539,246	44,568,186	Selenoprotein V	Not expressed*
EID-3	44,715,334	44,713,470	EID-2-like inhibitor of differentiation-3	
CRI2	44,721,289	44,722,664	CREBBP/EP300 inhibitor 2	
LOC390930	44,735,501	44,738,032	Similar to Eosinophil lysophospholipase (Charcot-Leyden crystal protein)	Pseudogene
LGALS13	44,785,004	44,789,955	Lectin, galactoside-binding, soluble, 13 (galectin 13)	Not expressed*
LOC441850	44,820,643	44,824,298	Similar to eosinophil lysophospholipase (Charcot-Leyden crystal protein)	Pseudogene
LOC148003	44,838,415	44,843,127	Similar to placental tissue protein 13 (placenta protein 13; galectin-13)	No mRNA/EST evidence
LOC400696	44,861,854	44,868,848	Eosinophil lysophospholipase-like	Not expressed*
LGALS14	44,877,533	44,891,927	Lectin, galactoside-binding, soluble, 14	Not expressed*
CLC	44,913,736	44,934,546	Charcot-Leyden crystal protein	Not expressed*
LOC342900	44,959,075	44,968,615	Hypothetical protein LOC342900	No mRNA/EST evidence
DYRK1B	45,007,831	45,016,681	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B	
FBL	45,016,938	45,028,813	Fibrillarin	
FCGBP	45,045,811	45,132,373	Fc fragment of IgG binding protein	
LOC440525	45,140,982	45,141,428	Proline-rich 13 pseudogene	Pseudogene
PSMC4	45,168,913	45,179,193	Proteasome (prosome, macropain) 26S subunit, ATPase, 4	
ZNF546	45,194,869	45,215,354	Zinc finger protein 546	
LOC390933	45,221,651	45,222,297	Similar to hypothetical protein	Pseudogene
LOC163131	45,248,079	45,232,104	Hypothetical protein LOC163131	Not evaluated
LOC284323	45,270,739	45,288,649	Hypothetical protein LOC284323	
MAP3K10	45,389,491	45,413,314	Mitogen-activated protein kinase kinase kinase 10	
TTC9B	45,413,805	45,416,138	Tetratricopeptide repeat domain 9B	Not expressed*
FLJ13265	45,419,955	45,424,404	Hypothetical protein FLJ13265	Not expressed*
LOC440526	45,429,392	45,430,177	Hypothetical protein LOC440526	
AKT2	45,431,556	45,483,036	v-akt murine thymoma viral oncogene homologue 2	
FLJ36888	45,518,813	45,546,262	Hypothetical protein FLJ36888	Not expressed*
PLD3	45,557,389	45,576,230	Phospholipase D family, member 3	

Abbreviation: EST, expressed sequence tag.
*Not expressed in PANC-1.

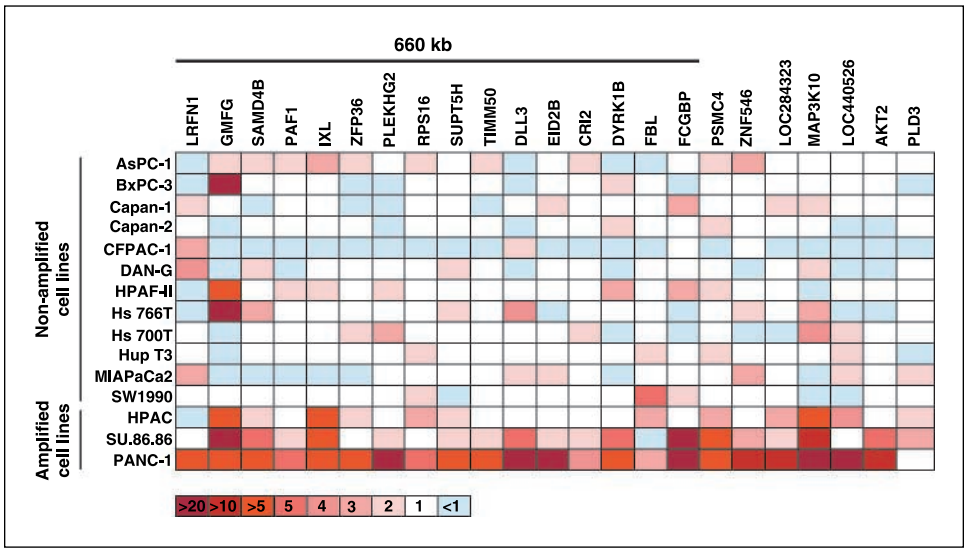

Figure 4. Schematic representation of mRNA expression levels of 23 genes within the 1.1 Mb amplicon at 19q13 in 16 pancreatic cancer cell lines. The expression levels were determined using quantitative real-time RT-PCR and were normalized against a housekeeping gene *TBP*. The relative expression levels for each gene were median centered and displayed as a pseudocolor gradient. The genes are arranged according to their chromosomal position (from centromere to telomere). *Bottom,* key to the color coding. *Horizontal line above the figure,* 660 kb amplicon core.

candidate genes from this region might provide a growth advantage for the cancer cells. Recently, two other genes from this region, *PAF1* and *DYRK1B*, were proposed to associate with pancreatic cancer development and cell survival, respectively (35, 36). Yet, again, our data do not show evidence that these genes would be the key targets of 19q13 amplicon.

In summary, our detailed characterization of the 19q13 amplicon in pancreatic cancer cell lines delineated a minimal region of amplification to a 1.1 Mb segment and further pinpointed a 660-kb amplification maximum. This amplicon was recognized in 19% of cell lines and 10% of primary pancreatic tumors. Expression profiling of genes residing in the amplicon revealed seven biologically interesting genes that were more strongly expressed in the amplified cell lines compared with the nonamplified ones. High-throughput loss-of-function screen by RNAi technology showed that down-regulation of *IXL*, and, to a lesser extent, *GMFG* and *SUPT5H*, resulted in decreased cell viability in the amplified PANC-1 but not in the nonamplified cells. Additionally, *IXL* knockdown was found to associate with G_0-G_1 arrest and increased apoptosis. Our results reveal *IXL* as a novel amplification

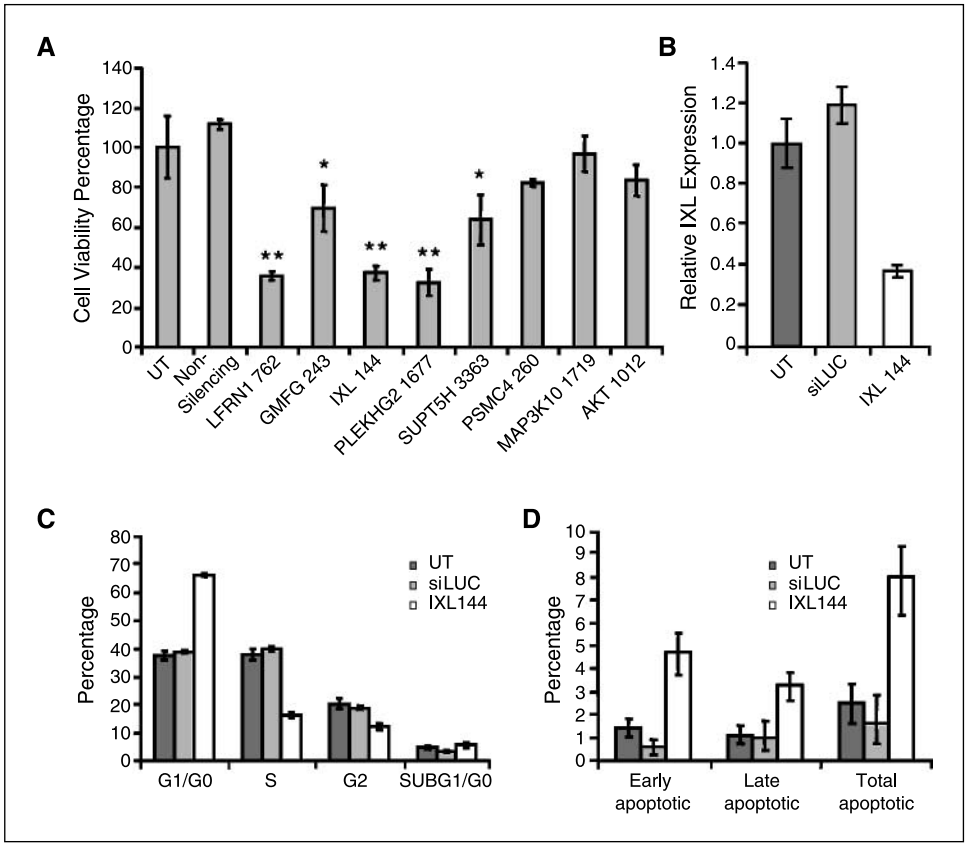

Figure 5. Functional evaluation of RNAi-based gene silencing in the PANC-1 cells. *A,* summary of data from the high-throughput RNAi viability screen. The number of cells were determined 96 h after siRNA transfection and compared with that of untreated control (*UT*). *Columns,* mean of four independent wells shown for selected genes as well as nonsilencing control siRNA; *bars,* SD. *, $P < 0.01$; **, $P < 0.001$, statistically significant reduction in cell viability. The experiment was repeated thrice with similar results. Raw data from the entire viability screen are shown in Supplementary Table S5. *B,* quantitative real-time RT-PCR analysis of *IXL* mRNA expression levels in PANC-1 cells 48 h after transfection of luciferase control siRNA (*siLUC*) or *IXL* 144 siRNA. Results were normalized against untreated cells. *Columns,* mean of triplicate experiments; *bars,* SD. *C, IXL* down-regulation results in G_0-G_1 arrest. Cell cycle analysis of untreated, luciferase siRNA–treated and *IXL* 144 siRNA–treated PANC-1 cells 48 h after transfection. *Columns,* mean of triplicate experiments; *bars,* SD. *D,* induction of apoptosis after *IXL* silencing. Percentage of early apoptotic, late apoptotic, and total apoptotic cells are shown for untreated, luciferase siRNA–treated, and *IXL* 144 siRNA–treated PANC-1 cells 48 h after transfection. *Columns,* mean of triplicate experiments; *bars,* SD.

target gene that is essential for the growth and survival of a subset of pancreatic carcinomas with 19q13 amplification. Thereby, *IXL* has a critical role in pancreatic cancer development and growth regulation and represents an ideal therapeutic target. However, it is possible that other genes in this region might also contribute to pancreatic cancer pathogenesis. Finally, this study shows that the combination of copy number and expression analysis together with targeted RNAi screen provides an efficient method for rapid identification of putative amplification target genes in cancer.

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