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LKW, a Putative Dual-specificity Kinase which is Down-regulated in Several Invasive Systems

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Abstract. *Using Affymetrix GeneChip® arrays, we have established the transcriptional profiles of two sublines of the human pancreatic adenocarcinoma cell lines SUIT-2, S2-007 (metastatic) and S2-028 (non-metastatic). By comparison of ESTs corresponding to differentially regulated mRNAs, we have identified the putative dual-specificity kinase LKW as down-regulated in the metastatic cell line S2-007. LKW is composed of 358 amino acids and contains catalytic domains corresponding to those of Ser/Thr- and Tyr-kinases. The gene encoding LKW consists of four exons separated by three introns and is located on chromosome 20p12.2-p13. In parallel LKW has been identified in different experimental settings by other groups and is referred to as NIPK, SKIP3 and TRB3. We noticed that the metastatic propensity of two additional pancreatic cellular systems correlates with the down-regulation of mRNA levels corresponding to LKW. Evaluation of a panel of mammary carcinoma cell lines scored as non-invasive and invasive based on an in vitro invasion assay indicated down-regulation of LKW mRNA levels in the invasive cell lines. mRNA for LKW was shown to be overexpressed in pancreatic carcinomas compared to normal pancreas and tissues derived from patients with chronic pancreatitis as well as in colon, kidney, breast and ovarian carcinomas compared to their matching normal tissues. Analysis of colorectal carcinomas covering Duke's stages A, B, C and D revealed down-regulation of LKW mRNA in specimens corresponding to Duke's stages C and D, representing invasive and metastatic stages compared to the less invasive stages corresponding to Duke's stages A and B. Our results indicate that down-regulation of mRNA encoding LKW correlates with an invasive status of mammary carcinoma cell lines and the metastatic propensity of colorectal carcinoma.*

The major reason for death of cancer patients is the dissemination of primary tumors, giving rise to distant metastases which are refractory to chemotherapy and radiotherapy (1). In order to metastasize, tumor cells must complete a series of steps including neovascularization and lymphangiogenesis, invasion of the host stroma, intravasation and extravasation of vessels of the blood and or lymphatic system and colonization of the parenchyma of distant organs. It has been shown that the vascular and the lymphatic system are connected allowing disseminated tumor cells to pass from one system to the other (2, 3).

Pancreatic cancer is the fifth leading cause of cancer deaths in industrialised countries and is one of the most difficult to treat. There is no valid screening test for this disease. Pancreatic cancer is rarely cured by surgery and is highly resistant to radiotherapy and chemotherapy. The majority of pancreatic cancers (>80 %) display a ductal epithelial phenotype and are highly aggressive with early local invasion and metastasis. The overall five-year survival rate is 0.4 %, with <10 % of patients alive after 12 months. Aspects of the pathogenesis of pancreatic ductal carcinoma, such as tumor invasion, tumor-stromal interaction, metastasis and resistance to chemotherapy are poorly understood. Therefore, improved diagnostic markers and molecular targets for therapy are urgently needed to increase the survival rate of pancreatic cancer patients by early diagnosis and target-based treatment (4-6).

In order to identify metastasis-associated genes, we have applied the Affymetrix transcriptional profiling technique (7-10) to a pair of human pancreatic tumor cell lines with high and low metastatic potential, respectively. Both cell lines are derived from SUIT-2, a human pancreatic cancer cell line established from a liver metastasis of a human pancreatic carcinoma (11). Subline S2-028 is non-metastatic,

Abbreviations: aa, amino acids; bp, base pairs; cDNA, complementary DNA; EST, Expressed Sequence Tag; kb, kilo base(s); MTE, Multiple Tissue Expression.

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whereas subline S2-007 exhibits high potential for metastasis to the lymph nodes and to the lung after subcutaneous injection (12). Our investigations resulted in the identification of the dual-specificity kinase LKW which is down-regulated in several invasive and metastatic systems. Dual-specificity kinases are able to phosphorylate both tyrosine and serine/threonine residues (13-16).

Materials and Methods

Human tissue samples and cell lines. Human pancreatic tissues from patients with histologically verified adenocarcinoma of the pancreas (n=6) or chronic pancreatitis (n=4) as well as control tissues from resection margins or organ donors (n=4) were collected by the Department of Internal Medicine I of the University of Ulm, Germany. Human normal colorectal epithelium (n=24) and primary cancers with different TNM stages (n=24) were provided by the Departments of Pathology, University of Regensburg and Hospital of Kassel, Germany. Human cancer cell lines were obtained from the following suppliers: SUIT-2 clones S2-007, S2-028, PATU-8988s, PATU-8988t (Department of Internal Medicine I, University of Ulm, Germany), BxPC-3, AsPC1 (European Collection of Cell Cultures, Salisbury, United Kingdom), MDA-MB-157, MDA-MB-175, MDA-MB-231, MDA-MB-330, MDA-MB-361, MDA-MB-436, MDA-MB-453, MDA-MB-468, MCF-7, Hs578T, BT-20, BT-474, BT483, BT-549, T47-D, ZR-75-1, ZR-75-30, CAMA-1, SK-BR-3, UCAA-812, Du4475 (R. Zeillinger, Department of Obstetrics and Gynecology, University of Vienna, Austria).

Affymetrix GeneChip® Profiling. Transcriptional profiling was performed as described earlier (17).

Northern blot analysis. Ten μg of total RNA from S2-007 and S2-028 cell lines were loaded side by side on a denaturing 1% agarose formaldehyde gel and size-separated by electrophoresis. Blotting to BrightStar-Plus™ positively-charged nylon membrane was done by capillary downward transfer. After UV-crosslinking (Stratagene UV Stratalinker 2400), the blot was hybridized to α-[^{32}P]dATP-labeled LKW cDNA with a specific activity of 2x10^9 cpm/μg using the Strip-EZ™ DNA Kit (Ambion Inc., Austin, Texas, USA). Pre-hybridization (30 min) and hybridization (overnight) with the radioactive probe was performed in ExpressHyb™ Hybridization Solution (Clontech, Palo Alto, CA, USA) at 68˚C. The membrane was washed in solution 1 (2x SSC, 0.05% SDS) at room temperature for 30-40 min with continuous agitation and several replacements of the wash solution 1 followed by a washing step with solution 2 (0.1 x SSC, 0.1% SDS) at 50˚C for 40 min with one change of fresh solution. The membrane was then exposed to Cronex, Medical X-Ray Films (Sterling Diagnostic Imaging Inc., USA) at -70˚C for 2 h. Equal loading and transfer of mRNA to the membrane was assessed by rehybridizing the blot with α-[^{32}P]dATP-labeled GAPDH cDNA probe.

Cloning of LKW cDNA. Based on the 447 bp fragment corresponding to EST Acc.-Nr. AI949781, 5'RACE PCR was performed as described in the 5'RACE System for Rapid Amplification of cDNA Ends Kit, Version 2.0 (Gibco BRL, Life Technologies, Carlsbad, CA, USA). First strand cDNA was synthesized from total RNA (treated with DNase I) using the gene-specific primer GSP1 (5'TCTCCTTTATTA GGCACAGG3') and SuperScript™ II, an Rnase H- derivative of the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MVL RT). After removal of unincorporated dNTPs and GSP1, TdT (Terminal deoxynucleotidyl transferase) was used to add homopolymeric tails to the 3' ends of the cDNA. Tailed cDNA then was amplified by PCR using a nested, gene-specific primer GSP2 (5'AGTATGGACCTGGGATTGTGGA3'), which anneals 3' to GSP1 and the deoxyinosine-containing abridged universal amplification primer AUAP (5'GGCCACGCGTCGACTAGTAC-3'). The enriched and purified cDNA was cloned into the PCR 2.1-TOPO-Vector (Invitrogen, San Diego, CA, USA) and sequenced.

Human Multiple Tissue Expression array (MTE™). The MTE™ array (Clontech) contains normalized loadings of poly A$^+$-RNA from 76 different tissues as well as control RNA's and DNA's as revealed in Figure 5. The blot was hybridized with an α-[^{32}P]dATP-labeled probe derived from LKW cDNA according to the instructions of the manufacturer and exposed to X-ray film at -70˚C for 26 and 30 h.

LightCycler® based PCR. LightCycler™–PCR was performed with a LightCycler® Instrument (Roche Molecular Biochemicals, Mannheim, Germany) in LightCycler® capillaries using a commercially available master mix containing Taq DNA polymerase, SYBR-Green I, deoxyribonucleoside triphosphates (LightCycler® DNA master SYBR-Green I, Roche Molecular Biochemicals). The PCR reaction was performed in a volume of 20 μl containing 2 μl of template cDNA, primers (forward 5'TCCACACACATGCAGTTCCT3'; reverse 5'AGGCCGACACTGGTACAAAG3'; final concentration: 0.5 μM), $MgCl_2$ (3 mM) and 2 μl of the master mix. Thirty-seven cycles of denaturation (95˚C for 1 sec), annealing (58˚C for 5 sec) and extension (72˚C for 8 sec) were performed. All temperature transition rates were set to 20˚C per sec. After completion of PCR amplification, a melting curve analysis was performed. For this procedure, PCR products were denatured at 95˚C, annealed at 65˚C and gradually heated to 95˚C, whereas SYBR®-Green I fluorescence was monitored stepwise every 0.1˚C. Each experiment was performed in duplicate. A calibration curve for calnexin was generated using serial dilutions (1:10, 1:50 and 1:80) of cDNA from the colon adenocarcinoma cell line KM12C. The relative amounts of LKW cDNA and calnexin were determined based on a calibration curve. Relative expression of the gene LKW was calculated as a normalized value. This was generated by dividing the steady-state levels of LKW mRNA and calnexin mRNA for each sample. The housekeeping gene calnexin was chosen because it is expressed at the same level in normal and neoplastic colorectal tissues. (Calnexin forward primer 5'ATTGTCAGATCG TTCATTGC3'; reverse primer 5'ATGGAACAGGTAACCA GCAT3').

TaqMan®based PCR. Real-time quantitative PCR was performed by means of TaqMan® technology and the ABI PRISM 7700 apparatus (Applied Biosystems, Foster City, CA, USA). Ten μg total RNA, isolated from frozen adenocarcinomas of the pancreas, chronic pancreatitis and normal pancreatic tissues, were used for reverse transcriptase reactions in a volume of 20 μl. The PCR reactions were then carried out by mixing 200 ng cDNA with 4μl of 10x SYBR-Green buffer, 3 mM $MgCl_2$, 1 mM dNTPs, 0.2 units Uracil-N Glycosylase, 1 unit AmpliTaq Gold, 4μl primer mix (300 nM each primer: forward 5'CCTGAGCACTAGGGCCTC3'; reverse 5'TGGCCACAGGACAGACCC3') in a final volume of 40 μl. PCR primers were designed to generate a DNA fragment of 50 bp using the Primer Express Software (PE Biosystems, Foster City, CA, USA).

The amplification cycles were as follows: 2 min at 50˚C followed by 10 min at 95˚C and 40 amplification cycles (95˚C for 15 sec and 60˚C for 60 sec). These experiments were performed twice. The results were calculated by subtraction of the steady-state levels of gene LKW and housekeeping gene xs13 for each sample. The value corresponding to the difference was divided by the steady-state level of the four healthy tissues. Ratios were squared and a reciprocal value was formed. (xs13for: 5'AGATCCGCATGTCCCTTC3'; xs13rev: 5'CCTTGCGCATCATGGTGTT3').

Bioinformatic tools. Translation of the LKW nucleotide sequence into the protein sequence was performed with the algorithm Translate. The programs Molecular Weight and Isoelectric from GCG (Genetics Computer Group, Inc., Madison, Wisc., USA) were used to predict the molecular weight and pI of the LKW protein.

The database 'Swissprot Human' was used for comparison with proteins of known structure. The serine/threonine protein kinase and tyrosine kinase homology domains were identified using the GCG motifs program searching in Prosite, database of protein sequence motifs and Pfam, a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains and families. The Ensembl Genome Browser software of The Wellcome Trust Sanger Institute and EMBL – EBI was used for the prediction of the genomic localization of gene LKW. The SIM4 computer program was used for the exon-intron-analysis of gene LKW.

Results

Identification of the cDNA of gene LKW and its genomic organization. Affymetrix transcriptional profiling experiments with the pancreatic tumor cell lines S2-007 and S2-028 indicated that the mRNA corresponding to EST accession number AI949781 is significantly down-regulated in the metastasizing cell line S2-007. These results were confirmed by Northern blotting, revealing a differentially expressed mRNA of 2.3 kb corresponding to the gene encoding LKW (Figure 1). The corresponding cDNA was cloned and sequenced as described in the 'Materials and Methods' section. The nt and aa sequences of the cDNA encoding LKW are displayed in Figure 2. The protein corresponding to LKW is composed of 358 aa. Database searches revealed putative catalytic domains corresponding to tyrosine kinases (aa 141 to 305) as well as to serine/threonine kinases (aa 80 to 315). The sequence of LKW therefore incorporates features of a dual-specificity kinase. A database search resulted in localization of the gene encoding LKW on chromosome 20p12.2-p13. According to the alignment produced by the program SIM4, the gene is composed of four exons (257 bp, 290 bp, 292 bp and 1361 bp) separated by three introns (6842 bp, 2986 bp and 4619 bp).

Homology of LKW with other dual-specificity kinases. As illustrated in Figure 3, we have identified five dual-specificity kinases with homology to the protein encoded by

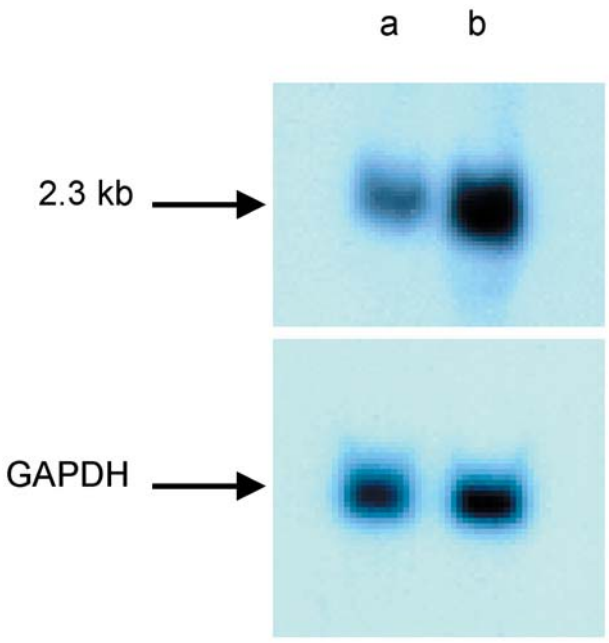


Figure 1. *Northern blot analysis of LKW RNA expressed in cell lines S2-007 and S2-028. Ten μg RNA derived from cell lines S2-007 and S2-028 were size-fractionated on a 1% agarose gel, transferred to a nylon-membrane and hybridized to α-[^{32}P] dATP-labeled probe specific for LKW prepared by PCR as described in the 'Materials and Methods' section. Lanes a and b correspond to RNA derived from cell lines S2-007 and S2-028, respectively.*

LKW. Figure 3 also displays subdomains with identical amino acids as well as single conserved aa. The catalytic domain of LKW shows the closest similarity with that of *S. cerevisae* SKIP (Acc.No. Q96RU8) (55%). The alignment of human LKW with human ERK1 and human ERK2 revealed 34% and 38% identity for amino acids corresponding to the catalytic domains. Searches of available DNA and protein data bases revealed no direct sequence similarities with any known gene or protein in addition to ERK1 and ERK2 (18, 19). ERK1 and ERK2 belong to the class of mitogen-activated protein kinases (MAP kinases), which are a group of closely related enzymes involved in several signal transduction pathways (20). The protein sequence of the catalytic domains of human dual-specificity kinase LKW was also 36% identical to human phosphotyrosine picked threonine kinase (PYT) and *S. cerevisiae* serine protein kinase (SPK1) (21). A previous study has demonstrated that PYT is associated with cell proliferation (22).

Expression of LKW mRNA in tumor cell lines with differing invasive and metastatic properties. Making use of the LightCycler® method, we have determined the steady-state level of LKW mRNA in several pancreatic carcinoma cell

Figure 2. *Nucleotide and amino acid sequence of the cDNA of LKW. Ser/Thr kinase homologous amino acids are covered by aa 80-315, Tyr kinase homologous aa correspond to aa 141-305. Ser/Thr kinase homologous aa are tinged with grey, Tyr kinase homologous aa are underlayered dark. The aa sequence of LKW was compared with proteins of known structure using the database Swissprot Human. Ser/Thr kinase and Tyr kinase homology was identified using the GCG (Genetics Computer Group, Inc.) motifs program by searching in PROSITE, database of protein sequence motifs, and Pfam, a large collection of multiple sequence alignments and Hidden Markov Models covering many common protein domains and families.*

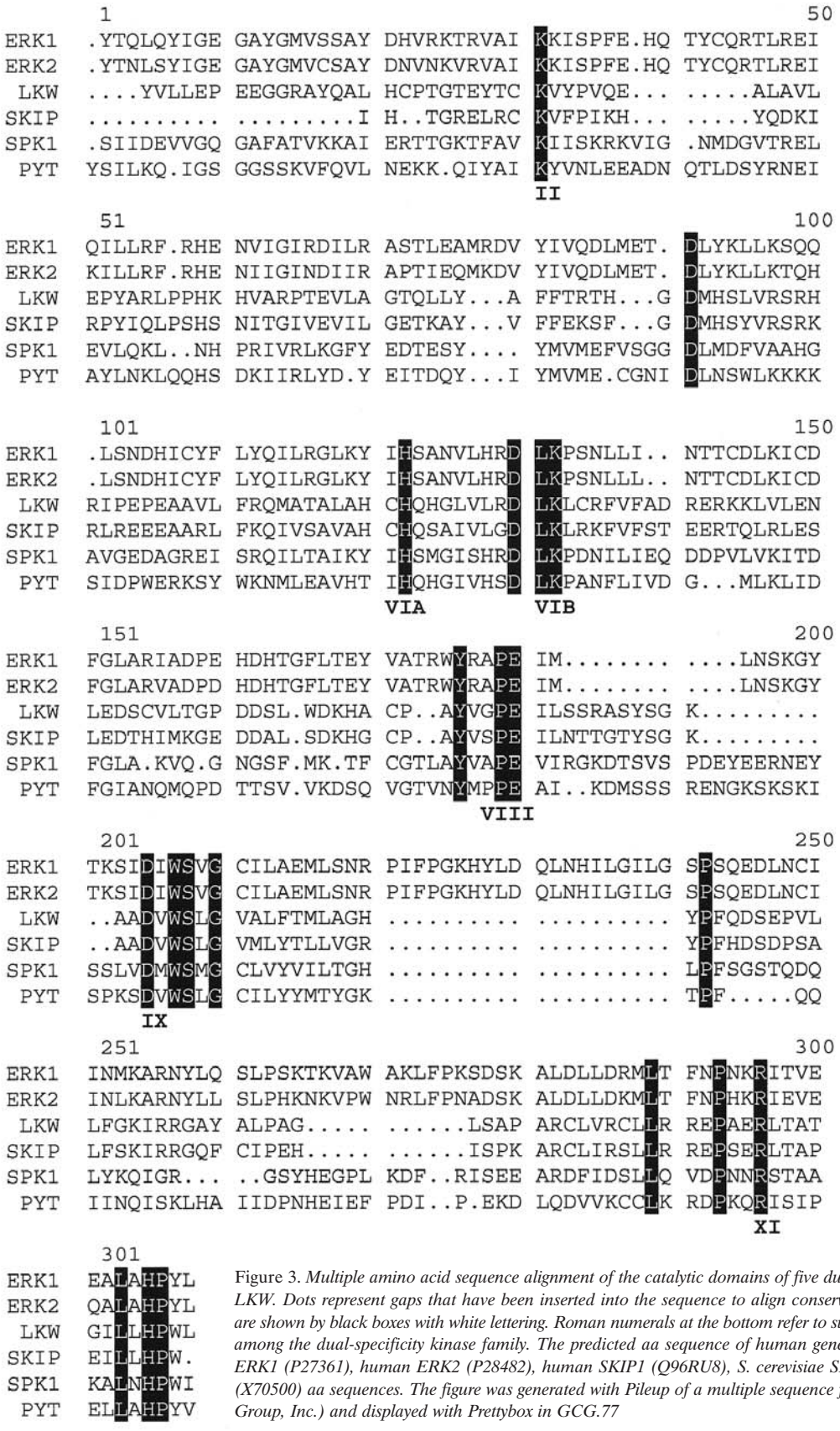


Figure 3. *Multiple amino acid sequence alignment of the catalytic domains of five dual-specificity protein kinases and LKW. Dots represent gaps that have been inserted into the sequence to align conserved regions. Highly conserved aa are shown by black boxes with white lettering. Roman numerals at the bottom refer to subdomains or single aa conserved among the dual-specificity kinase family. The predicted aa sequence of human gene LKW was aligned with human ERK1 (P27361), human ERK2 (P28482), human SKIP1 (Q96RU8), S. cerevisiae SPK1 (M55623) and human PYT (X70500) aa sequences. The figure was generated with Pileup of a multiple sequence file in GCG (Genetics Computer Group, Inc.) and displayed with Prettybox in GCG.77*

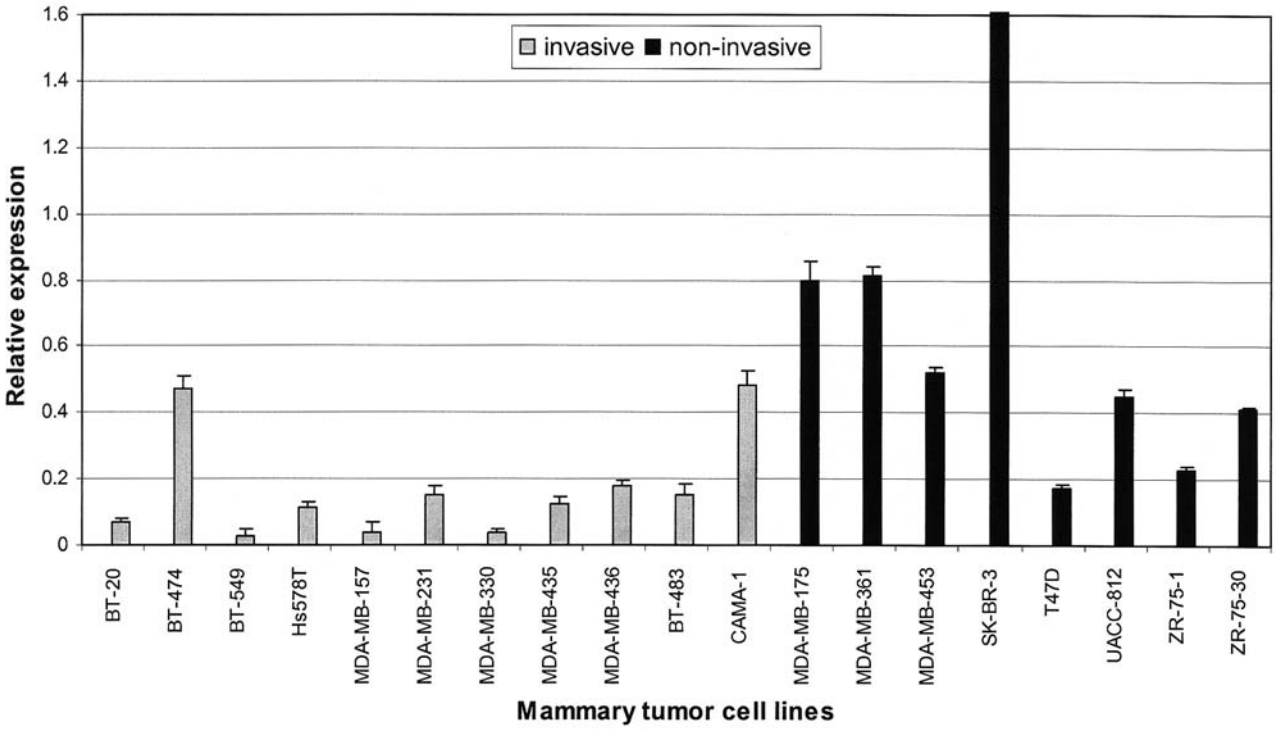
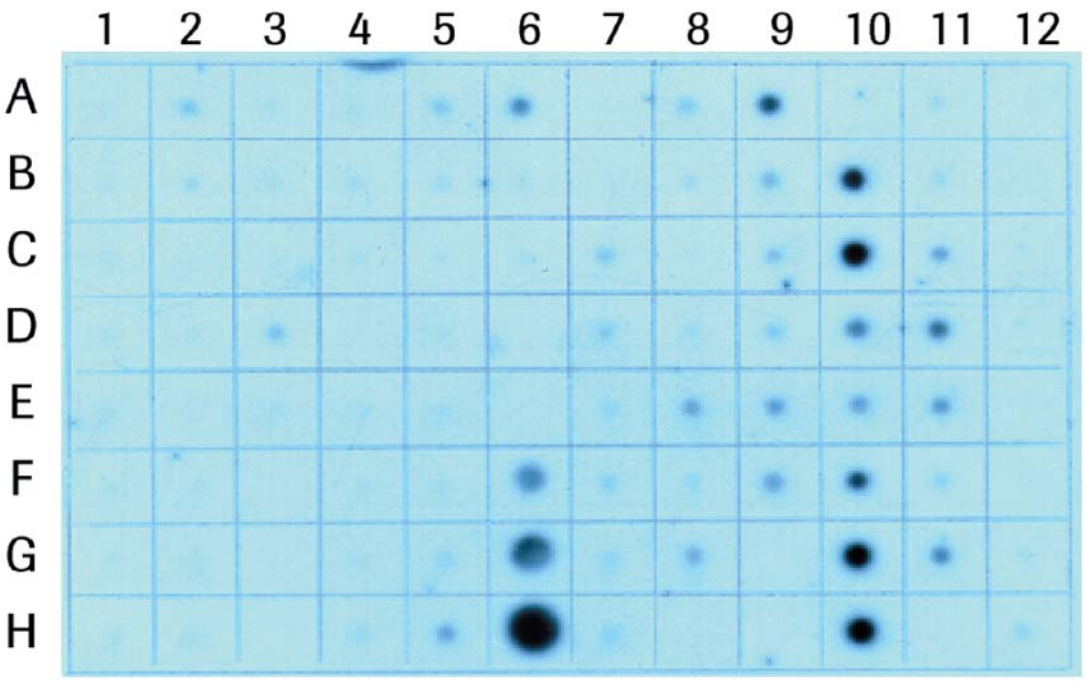


Figure 4. *Relative expression of LKW mRNA in invasive and non-invasive mammary tumor cell lines by LightCycler® PCR. One μg DNAse I-treated total RNA of 19 mammary tumor cell lines was used for LightCycler® PCR as described in the 'Materials and Methods' section. Mammary tumor cell lines with invasive and non-invasive potential were examined. Results are derived from two independent experiments and were calculated as the ratio between LKW mRNA and calnexin mRNA expression for each sample.*

lines with different metastatic properties (S2-007, S2-028, PaTu 8988s, PaTu 8988t, AsPC and BxPC-3). The two cell lines PaTu 8988s and PaTu 8988t exhibit a different grade of differentiation and are derived from a primary human pancreatic adenocarcinoma (23). PaTu 8988s metastasizes to the lungs, whereas PaTu 8988t is not metastatic. The pancreatic tumor cell line AsPC1 (24) exhibits a high potential for metastasis. Cell line BxPC-3 is non-metastatic (24, 25). We note a 10-fold increased steady-state level of LKW mRNA in the non-metastasizing cell line S2-028 compared to the metastasizing S2-007 cell line, a one and a half fold increase in cell line PaTu 8988t compared to PaTu 8988s and a 2.5-fold increase in cell line BxPC-3 compared to cell line AsPC1 (data not shown). We have extended this type of analysis to several mammary carcinoma cell lines which were scored for their invasive properties with an *in vitro* invasion assay (26, 27). The following cell lines were scored as invasive: BT-20, BT 474, BT 549, Hs 578T, MDA-MB 157, MDA-MB 231, MDA-MB-330, MDA-MB 435 and MDA-MB-436. The following cell lines were scored as non-invasive: BT 483, CAMA-1, MDA-MB-175, MDA-MB-361, MDA-MB-453, SK-BR-3, T47D, UCAA-812, ZR-75-1 and ZR-75-30. Comparison of the average expression level of LKW mRNA of the non-invasive *versus* the invasive cell lines indicates a four-fold increase in the non-invasive cell lines (Figure 4).

Expression pattern of mRNA for LKW in normal and tumor tissues and tumor cell lines. MTE blot analysis (Figure 5) points to a tissue-restricted expression pattern of the mRNA encoding LKW. Most prominent signals were found in tissues such as liver and colon transverse, weaker signals were noticed in organs such as colon ascending, prostate, pancreas, salivary gland and mammary gland. Also, fetal tissues derived from kidney, liver, spleen and lung scored positive for LKW mRNA. However, the strongest signals were found in tumor cell lines, with prominent signals in HeLa S3, leukemia cell line K-562, Burkitt's lymphoma cell line Daudi, colorectal adenocarcinoma cell line SW480 and lung carcinoma cell line A540. Weaker signals were found in leukemia cell line MOLT-4 and Burkitt's lymphoma cell line Raji.

We also analyzed LKW mRNA levels in a larger number of different normal and tumor tissues making use of a cancer profiling array, which contains normalized cDNA from 241 tumor and corresponding normal tissues from individual patients. After hybridization of this array with a LKW cDNA probe, we found a significant up-regulation of LKW mRNA in colon-, kidney-, breast- and ovary carcinomas compared to



	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebellum, left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain	yeast total RNA
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney	*E. coli* rRNA
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver	*E. coli* DNA
E	occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen	Poly r(A)
F	temporal lobe	hippo-campus		ventricle, right	ilocecum		lymph node	testis	mammary gland	Burkitt's lymphoma, Daudi	fetal thymus	human C_0t-1 DNA
G	p. g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone marrow	ovary		colorectal adeno-carcinoma SW480	fetal lung	human DNA 100 ng
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549		human DNA 500 ng

* paracentral gyrus

Figure 5. *Expression analysis of LKW mRNA in human tissues and tumor cell lines. The Multiple Tissue Array was hybridized with an α-[^{32}P] dATP-labeled probe derived from the LKW cDNA. F6 corresponds to 1 μg total RNA, G6 to 5 μg total RNA and H6 to 10 μg total RNA from cell line S2-007. The code is revealed below.*

the corresponding normal tissues. However, in the majority of the other tissue types, no notable differences between normal and tumor tissue were detected (data not shown).

Expression of mRNA for gene LKW in pancreas-related tissues. We determined the steady-state levels of LKW mRNA in four normal pancreatic tissues, four pancreatic tissues derived from

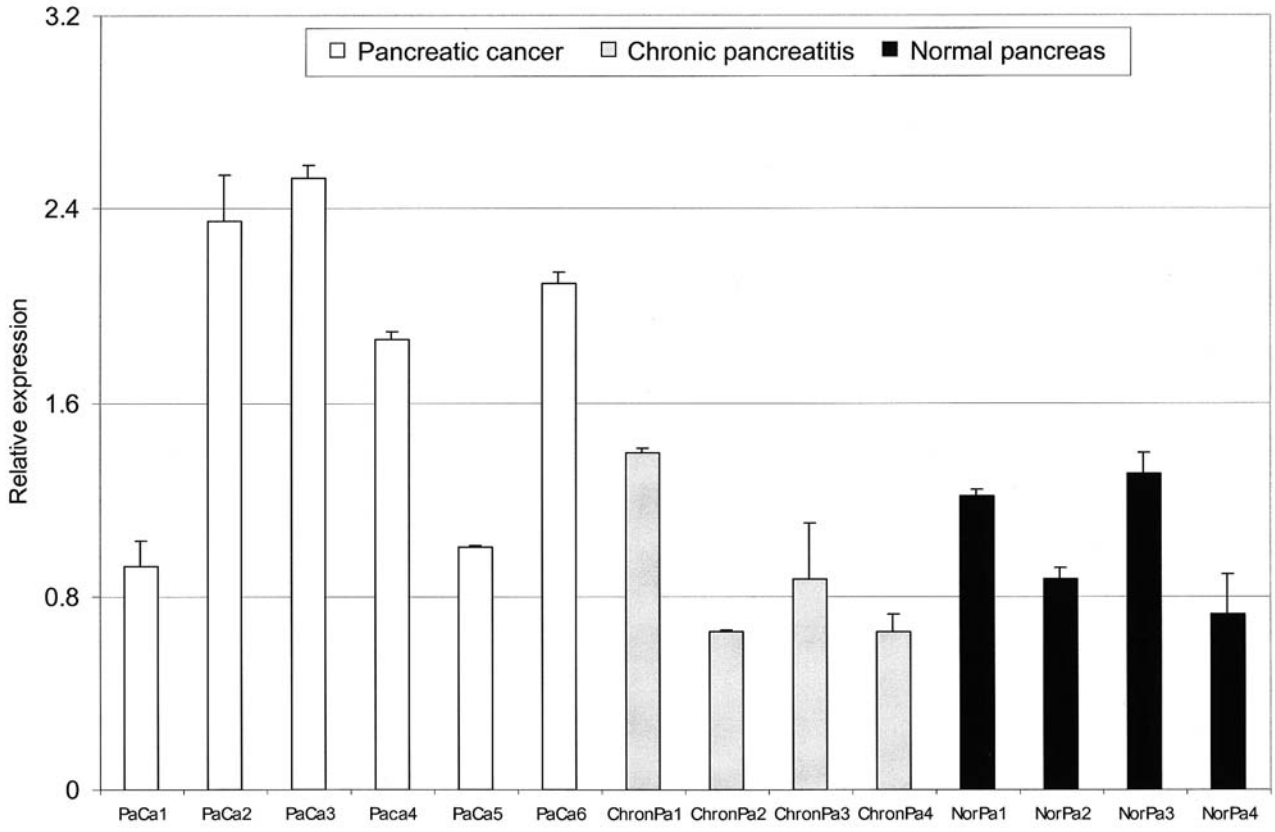


Figure 6. *Relative expression of LKW mRNA in samples derived from adeno-carcinomas of the pancreas, chronic pancreatitis and pancreatic control tissues. Ten μg total RNA was used for reverse transcriptase reactions. These experiments were performed twice. The results were calculated by subtraction of steady-state levels of LKW mRNA and housekeeping gene xs13 mRNA for each sample and subsequently the difference was divided by the steady-state level of LKW mRNA of the four healthy tissues. Ratios were squared and a reciprocal value was formed.*

patients with chronic pancreatitis and six patients with ductal pancreatic carcinoma making use of the TaqMan® real-time PCR (Figure 6). We did not detect a significant difference of the steady state level of mRNA for gene LKW in normal *versus* pancreatic tissues derived from patients with chronic pancreatitis. In two of the tissues derived from pancreatic carcinoma, the steady-state level of mRNA for gene LKW was not increased compared to normal pancreatic tissue, in four tumor-derived tissues we noted a 1.4-fold to 2.0-fold increase of the steady-state level of LKW mRNA.

Expression of LKW mRNA in colon carcinoma tissues with different staging. Making use of the LightCycler technology, we compared the steady-state levels of LKW mRNA in 24 colon cancer tissues corresponding to different staging and 24 matching normal mucosa tissues (Figure 7). The tumor tissues were classified as: Duke's A (4), Duke's B (4), Duke's C (8) and Duke's D (8). We noted dramatic overexpression of LKW mRNA (up to 400-fold) in tumor tissues *versus* normal colonic mucosa. We have averaged the relative expression levels of LKW mRNA corresponding to Duke's stages A, B, C and D. This resulted in the following relative expression levels of LKW mRNA: Duke's A=1, Duke's B=1.9, Duke's C=0.4 and Duke's D=0.2.

Discussion

We have identified LKW, a dual-specificity kinase composed of 358 aa. The corresponding gene is located on chromosome 20p12.2-p13 with four exons separated by three introns. Database searches and amino acid comparisons have revealed significant sequence similarity to diverse members of the family of dual-specificity kinases. *S. cerevisae* SKIP (Acc.No. Q96RU8) displayed the greatest catalytic domain similarity to LKW (55%). Other molecules with a high degree of similarity include human ERK1 (34 %) and ERK2 (38 %), human SPK1 (36%) and human PYT (36%) (Figure 3). PYT is expressed in rapidly proliferating cells, indicating association of this protein with cell proliferation. The functions of SKIP, SPK1 and PYT are still unknown. Dual-

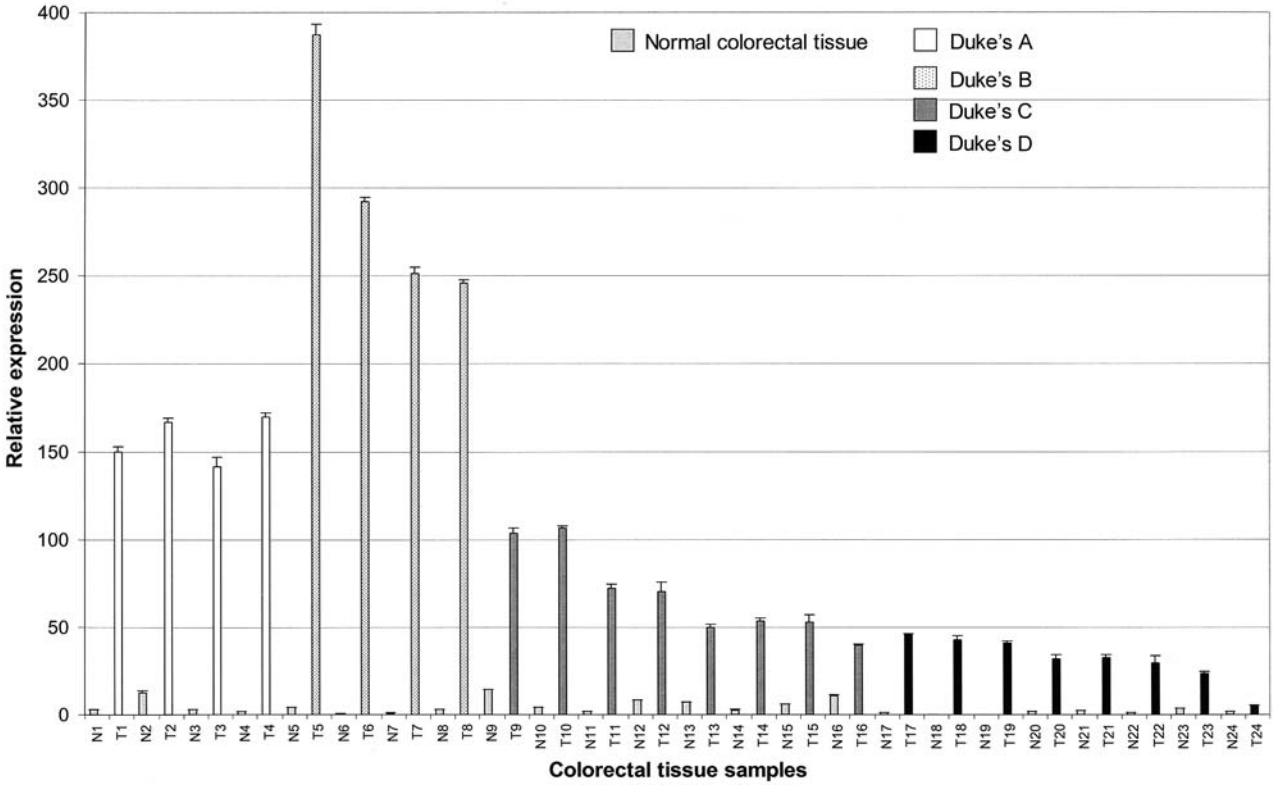


Figure 7. *Expression of LKW mRNA in normal mucosa and different stages of colon carcinoma. Expression of LKW mRNA was determined by LightCycler® PCR. The relative amounts of LKW cDNA and calnexin were determined on the basis of an established calibration curve. Relative expression of LKW mRNA was calculated as a normalized value. This was generated by dividing the steady-state levels of LKW mRNA and calnexin mRNA for each sample. The acronym N refers to normal tissue and the acronym T to tumor tissue. Notations T1 - T4 represent tumors categorized as Duke's stage A, T5 – T8 represent Duke's stage B tumors, T9 – T16 represent Duke's stage C tumors and notations T17 – T24 correspond to Duke's stage D tumors. Results are derived from two independent experiments. Data were calculated as the average of two independent experiments.*

specificity kinases are able to phosphorylate both Ser/Thr as well as Tyr-residues (16). Dual specificity kinases play a central role in the 'kinase cascade' that regulates pathways controling proliferation, apoptosis and differentiation. A prototypical kinase module consists of a minimum of three kinases, an upstream Ser/Thr kinase, middle dual-specificity kinase and a downstream Ser/Thr kinase. Recombinant expression of LKW is a prerequisite for further characterization of the enzymatic properties of LKW and to define the subclass of dual-specific kinase into which LKW can be integrated. Three subclasses have been defined: kinases that phosphorylate Ser-Thr and Tyr-residues of exogenous substrates, kinases that exhibit dual-specificity due to autophosphorylation and kinases that possess the structural motif characteristic for dual-specificity kinases (16).

One of the features of the newly described dual-specificity kinase is its down-regulation in invasive and metastasizing pancreatic carcinoma cells at the transcriptional level (Figure 1). In pancreatic carcinoma, we noted significantly increased levels of LKW mRNA in comparison to tissues derived from normal pancreas and tissues derived from patients with chronic pancreatitis (Figure 6). We were not able to establish a correlation between LKW mRNA expression and tumor progression because surgically removed pancreatic carcinoma almost always corresponds to late stage carcinoma. As shown in Figure 4, in mammary carcinoma cell lines, down-regulation of LKW mRNA correlates with invasiveness in *in vitro* assays (26). It was shown that the non-invasive cell lines score as ER^+ and $PAI\text{-}1^-$ and the invasive ones were categorized as ER^- and $PAI\text{-}1^+$ (26). High steady-state levels of LKW mRNA were found in colon, liver, pancreas, prostate, salivary gland and mammary gland (Figure 5). Colon-, kidney-, breast- and ovarian carcinomas revealed up-regulation of LKW mRNA compared to corresponding normal tissues (data not shown). Another outcome of our investigations is the demonstration of decreased mRNA expression for LKW in colon cancer Duke's C and D specimen representing cancer stages with tumor cell dissemination compared to those classified as Duke's A and B representing stages with no or infrequent dissemination

(Figure 7). In order to investigate the correlation between expression of LKW mRNA with metastasis of colorectal cancer, we intend to study the expression of LKW in matching specimens of primary cancer and its derived liver metastases.

While our work was in progress the gene and cDNA corresponding to LKW was identified in different experimental settings by different groups and is referred to as NIPK, TRB3 and SKIP3 (30-32).NIPK may play a role in a common pathway leading to programmed neuronal cell death and may serve as an endogenous antagonist competing for substrate with other kinases that act to promote neuronal cell survival (30). However, no conclusive experimental evidence is presented. NIPK also inhibits NF-kappa B signaling with its subunit p65. The encoded protein can sensitize cells to TNF- and TRAIL-induced apoptosis. TBR3 was identified as a tribble homolog that inhibits Akt/PKB activation in liver (31).

An oncological context was highlighted with identification of SKIP3 which is overexpressed in multiple human tumors and regulated by hypoxia (32). It was speculated that SKIP3 acts as an important participant in tumor cell growth. We also found overexpression of LKW in tumors such as colon, kidney, breast and ovarian carcinomas and in pancreatic carcinomas (Figure 6). However, as outlined in the previous section we note an inverse correlation between invasion and progression and the expression of LKW. Since the functional properties of LKW (alias NIPK, TBR3 and SKIP3) might be cell-type specific, it is presently not possible to explain the correspondence of LKW down-regulation and invasion and progression of tumor from a mechanistic point of view.

Identification of substrates of LKW by immunoprecipitation experiments and by making use of the yeast two-hybrid system will be a first step in elucidating the pathway(s) in which LKW is involved and thus defining the steps of the metastatic cascade in which LKW might play a role. Furthermore, as soon as polyclonal sera and monoclonal antibodies directed against LKW are available, we will investigate the relationship between expression of LKW and metastasis by immunohistochemistry of primary tumors and metastatic lesions of different tumor subtypes making use of tissue microarrays (28, 29).

Manzano *et al.* have shown that CL100, a dual-specificity phosphatase, is down-regulated in advanced epithelial ovarian cancer and its re-expression decreases its malignant potential (30). One of our next steps will therefore be the establishment of S2-007 cell clones stably overexpressing LKW for functional characterization of the gene product in invasion assays and *in vivo* xenograft models.

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