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Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes

Andrew V. Biankin[1,2,3], Nicola Waddell[4], Karin S. Kassahn[4], Marie-Claude Gingras[5,6], Lakshmi B. Muthuswamy[7], Amber L. Johns[1], David K. Miller[4], Peter J. Wilson[4], Ann-Marie Patch[4], Jianmin Wu[1], David K. Chang[1,2,3], Mark J. Cowley[1], Brooke B. Gardiner[4], Sarah Song[4], Ivon Harliwong[4], Senel Idrisoglu[4], Craig Nourse[4], Ehsan Nourbakhsh[4], Suzanne Manning[4], Shivangi Wani[4], Milena Gongora[4], Marina Pajic[1], Christopher J. Scarlett[1,8], Anthony J. Gill[1,9,10], Andreia V. Pinho[1], Ilse Rooman[1], Matthew Anderson[4], Oliver Holmes[4], Conrad Leonard[4], Darrin Taylor[4], Scott Wood[4], Qinying Xu[4], Katia Nones[4], J. Lynn Fink[4], Angelika Christ[4], Tim Bruxner[4], Nicole Cloonan[4], Gabriel Kolle[11], Felicity Newell[4], Mark Pinese[1], R. Scott Mead[1,12], Jeremy L. Humphris[1], Warren Kaplan[1], Marc D. Jones[1], Emily K. Colvin[1], Adnan M. Nagrial[1], Emily S. Humphrey[1], Angela Chou[1,12], Venessa T. Chin[1], Lorraine A. Chantrill[1], Amanda Mawson[1], Jaswinder S. Samra[9,13], James G. Kench[1,10,14], Jessica A. Lovell[1], Roger J. Daly[1], Neil D. Merrett[2,15], Christopher Toon[1], Krishna Epari[16], Nam Q. Nguyen[17], Andrew Barbour[18], Nikolajs Zeps[19], Australian Pancreatic Cancer Genome Initiative*, Nipun Kakkar[5], Fengmei Zhao[5], Yuan Qing Wu[5], Min Wang[5], Donna M. Muzny[5], William E. Fisher[6,20], F. Charles Brunicardi[21], Sally E. Hodges[6,20], Jeffrey G. Reid[5], Jennifer Drummond[5], Kyle Chang[5], Yi Han[5], Lora R. Lewis[5], Huyen Dinh[5], Christian J. Buhay[5], Timothy Beck[7], Lee Timms[7], Michelle Sam[7], Kimberly Begley[7], Andrew Brown[7], Deepa Pai[7], Ami Panchal[7], Nicholas Buchner[7], Richard De Borja[7], Robert E. Denroche[7], Christina K. Yung[7], Stefano Serra[22], Nicole Onetto[7], Debabrata Mukhopadhyay[23], Ming-

Correspondence and requests for materials should be addressed to S.M.G. (s.grimmond@imb.uq.edu.au)..
*Lists of participants and their affiliations appear below.
‡Deceased.

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Author Information BAM files and associated metadata in XML format have been uploaded to the European Genome-phenome Archive (EGA; http://www.ebi.ac.uk/ega) under accession numbers EGAS00001000154 and EGAS00001000343. Additional sequence data is located at dbGAP accession number phs000516.v1.p1. Reprints and permissions information is available at www.nature.com/reprints. Readers are welcome to comment on the online version of the paper.

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Sound Tsao[22], **Patricia A. Shaw**[22], **Gloria M. Petersen**[23], **Steven Gallinger**[22,24], **Ralph H. Hruban**[25], **Anirban Maitra**[25], **Christine A. Iacobuzio-Donahue**[25], **Richard D. Schulick**[26], **Christopher L. Wolfgang**[26], **Richard A. Morgan**[25], **Rita T. Lawlor**[27], **Paola Capelli**[28], **Vincenzo Corbo**[27], **Maria Scardoni**[28], **Giampaolo Tortora**[29], **Margaret A. Tempero**[30], **Karen M. Mann**[31], **Nancy A. Jenkins**[31], **Pedro A. Perez-Mancera**[32], **David J. Adams**[33], **David A. Largaespada**[34], **Lodewyk F. A. Wessels**[35], **Alistair G. Rust**[33], **Lincoln D. Stein**[7], **David A. Tuveson**[32], **Neal G. Copeland**[31], **Elizabeth A. Musgrove**[1,36], **Aldo Scarpa**[27,28], **James R. Eshleman**[25], **Thomas J. Hudson**[7], **Robert L. Sutherland**[1,36,‡], **David A. Wheeler**[5], **John V. Pearson**[4], **John D. McPherson**[7], **Richard A. Gibbs**[5], and **Sean M. Grimmond**[4]

[1]The Kinghorn Cancer Centre, 370 Victoria Street, Darlinghurst, and the Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney, New South Wales 2010, Australia. [2]Department of Surgery, Bankstown Hospital, Eldridge Road, Bankstown, Sydney, New South Wales 2200, Australia. [3]South Western Sydney Clinical School, Faculty of Medicine, University of New South Wales, Liverpool, New South Wales 2170, Australia. [4]Queensland Centre for Medical Genomics, Institute for Molecular Bioscience, The University of Queensland, St Lucia, Brisbane, Queensland 4072, Australia. [5]Department of Molecular and Human Genetics, Human Genome Sequencing Center, Baylor College of Medicine, One Baylor Plaza, MS226, Houston, Texas 77030-3411, USA. [6]Michael E. DeBakey Department of Surgery, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030-3411, USA. [7]Ontario Institute for Cancer Research, Toronto M5G 0A3, Canada. [8]School of Environmental & Life Sciences, University of Newcastle, Ourimbah, New South Wales 2258, Australia. [9]Department of Anatomical Pathology, Royal North Shore Hospital, St Leonards, Sydney, New South Wales 2065, Australia. [10]University of Sydney, Sydney, New South Wales 2006, Australia. [11]Life Technologies, Brisbane, Queensland 4000, Australia. [12]Department of Anatomical Pathology, Sydpath, St Vincent's Hospital, Sydney, New South Wales 2010, Australia. [13]Department of Surgery, Royal North Shore Hospital, St Leonards, Sydney, New South Wales 2065, Australia. [14]Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Camperdown, New South Wales 2050, Australia. [15]School of Medicine, University of Western Sydney, Penrith, New South Wales 2175, Australia. [16]Department of Surgery, Fremantle Hospital, Alma Street, Fremantle, Western Australia 6160, Australia. [17]Department of Gastroenterology, Royal Adelaide Hospital, North Terrace, Adelaide, South Australia 5000, Australia. [18]Department of Surgery, The University of Queensland, Princess Alexandra Hospital, Ipswich Road, Woollongabba, Queensland 4102, Australia. [19]School of Surgery M507, University of Western Australia, 35 Stirling Hwy, Nedlands 6009, Australia and St John of God Pathology, 12 Salvado Road, Subiaco, Western Australia 6008, USA. [20] The Elkins Pancreas Center at Baylor College of Medicine, 6620 Main Street, Suite 1450, Houston, Texas 77030, USA. [21]David Geffen School of Medicine at UCLA, Los Angeles, Callfornia 90024, USA. [22]University Health Network, Toronto M5G 2C4, Canada. [23]Mayo Clinic, Rochester, Minnesota 55905, USA. [24]Mount Sinai Hospital, Toronto M5G 1X5, Canada. [25]Department of Pathology, The Sol Goldman Pancreatic Cancer Research Center, the Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, USA. [26]Department of Surgery, The Sol Goldman Pancreatic Cancer Research Center, the Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, USA. [27]ARC-NET center for applied research on cancer, University and Hospital Trust of Verona, Verona 37134, Italy. [28]Department of Pathology and Diagnostics, University of Verona, Verona 37134, Italy. [29]Department of Surgery and Oncology, University and Hospital Trust of Verona, Verona 37134, Italy. [30]Division of Hematology and Oncology, University of California, San Francisco 94115, USA. [31]Cancer Research Program, The Methodist Hospital Research Institute, 6670 Bertner Avenue, Houston, Texas 77030, USA. [32]Li Ka Shing Centre, Cambridge Research Institute, Cancer Research UK, and Department of Oncology, Robinson Way, Cambridge CB2 0RE, UK. [33]Experimental Cancer Genetics, Wellcome Trust Sanger Institute, Hinxton CB10 1HH, UK. [34]Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota 55455, USA.

[35]Bioinformatics and Statistics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. [36]St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, New South Wales 2010, Australia.

Abstract

Pancreatic cancer is a highly lethal malignancy with few effective therapies. We performed exome sequencing and copy number analysis to define genomic aberrations in a prospectively accrued clinical cohort (n = 142) of early (stage I and II) sporadic pancreatic ductal adenocarcinoma. Detailed analysis of 99 informative tumours identified substantial heterogeneity with 2,016 non-silent mutations and 1,628 copy-number variations. We define 16 significantly mutated genes, reaffirming known mutations (*KRAS, TP53, CDKN2A, SMAD4, MLL3, TGFBR2, ARID1A* and *SF3B1*), and uncover novel mutated genes including additional genes involved in chromatin modification (*EPC1* and *ARID2*), DNA damage repair (*ATM*) and other mechanisms (*ZIM2, MAP2K4, NALCN, SLC16A4* and *MAGEA6*). Integrative analysis with *in vitro* functional data and animal models provided supportive evidence for potential roles for these genetic aberrations in carcinogenesis. Pathway-based analysis of recurrently mutated genes recapitulated clustering in core signalling pathways in pancreatic ductal adenocarcinoma, and identified new mutated genes in each pathway. We also identified frequent and diverse somatic aberrations in genes described traditionally as embryonic regulators of axon guidance, particularly SLIT/ROBO signalling, which was also evident in murine Sleeping Beauty transposon-mediated somatic mutagenesis models of pancreatic cancer, providing further supportive evidence for the potential involvement of axon guidance genes in pancreatic carcinogenesis.

Pancreatic cancer is the fourth leading cause of cancer death, with an overall 5-year survival rate of <5%, statistics that have not changed in almost 50 years[1]. Advances in neoadjuvant and adjuvant chemotherapeutic regimens have resulted in some improvement in outcome, but pancreatectomy remains the single most effective treatment modality for pancreatic cancer, and offers the only potential for cure. Only 20% of patients present with localized, non-metastatic disease which is suitable for resection[2]. Those who undergo resection and receive adjuvant therapy have a median survival of 12–22 months and a 5-year survival of 20–25%[3]. Existing systemic therapies are only modestly effective and the median survival for patients with metastatic disease remains 6 months. Genomic characterization of pancreatic ductal adenocarcinoma (PDAC), which accounts for over 90% of pancreatic cancer, has so far focused on targeted polymerase chain reaction (PCR)-based exome sequencing of primary and metastatic lesions propagated as xenografts or cell lines[4]. A deeper understanding of the underlying molecular pathophysiology of the clinical disease is needed to advance the development of effective therapeutic and early detection strategies.

Clinical cohort

A cohort of 142 consecutive patients with primary operable, untreated PDAC who underwent pancreatectomy with curative intent (pre-operative clinical stages I and II) were recruited, and consent was obtained for genomic sequencing through the Australian Pancreatic Cancer Genome Initiative (APGI), the Baylor College of Medicine Pancreatic Cancer Genome Project and the Ontario Institute for Cancer Research Pancreatic Cancer Genome Study (ABO collaboration) between June 2005 and June 2011 as part of the International Cancer Genome Consortium (ICGC)[5]. Detailed clinico-pathological characteristics of the cohort demonstrated features typical of resected PDAC with regard to tumour size, grade, lymph node metastasis and survival when compared to multiple retrospectively acquired cohorts[6–8], defining the accrued population as representative of the clinical disease in the community (Supplementary Table 1 and Supplementary Fig. 1).

Cellularity and mutation detection

A major challenge in genomic sequencing is the low malignant epithelial cell content of many cancers, which can adversely impact on the sensitivity of mutation detection. Most sequencing studies so far have used samples with >70% tumour cellularity, or cell lines/ xenografts[4,9]. To implement genomic sequencing approaches in clinical practice, it is imperative to efficiently and accurately detect actionable mutations in diagnostic clinical samples. We devised methodologies to overcome the challenges associated with extensive desmoplastic stroma that is characteristic of the majority of PDAC, and these strategies facilitated the discovery of novel molecular mechanisms in the pathophysiology of this disease. The cellularity of each primary sample was estimated through pathological review, deep amplicon-based sequencing of exons 2 and 3 of *KRAS* (average depth of 1,000×), and single nucleotide polymorphism (SNP) array-based cellularity estimates using a novel algorithm (qpure)[10]. *KRAS* mutations were identified in 93% of 142 cases and tumour cellularity ranged from 5% to 85% with a mean of 38% (Supplementary Table 2, Supplementary Figs 2 and 3, and Supplementary Methods).

To inform cellularity thresholds for subsequent analyses, we defined the impact of stromal DNA content on mutation detection by exome capturing and sequencing different mixtures of cancer cell line and matched germline DNA (100%, 80%, 60%, 40%, 20% and 10% cell line DNA) when sequenced to a depth of 70× coverage. Using these data as a standard, the median sensitivity to detect true positives across all samples in the cohort with greater than 20% epithelial cellularity was estimated at 45% (Supplementary Table 3). An informative cohort of 99 patients who had greater than 20% cellularity and/or ≥10 validated somatic mutations was taken forward for further analysis.

Mutation detection and CNV analysis

We performed hybrid-selection-based capture and sequencing of the entire exomes of tumour and matched normal DNA derived from all 142 patients using a combination of capture systems and next-generation sequencing platforms (see Supplementary Methods). The sequence depths at each site (APGI 65×, BCM 104× and OICR 205×) were adopted to ensure suitable sensitivity across their respective cohorts (Supplementary Table 3). In the informative 99 samples, we detected 2,627 high-confidence mutations, 2,016 of which were non-silent (Table 1). A total of 1,502 of these events (1,350 non-silent) were independently validated via an orthogonal sequencing method (see Supplementary Methods). The average number of mutations detected per patient was 26 (range 1–116), consistent with the expected sensitivity based on cellularity estimates and previous studies[4,11] (Supplementary Table 2). We confirmed the high prevalence of genetic aberrations known to be important in PDAC and observed mutations in 38 of the 79 genes (48% overlap) that occurred more than once previously reported by ref. 4, and 186 of all 998 mutated genes (19% overlap) in that study. We also defined a large number of novel mutations (1,456 genes), most of which occurred at low frequency (see Supplementary Tables 4– 6 and Supplementary Fig. 4 for detailed comparisons). The observed transversion/transition rates in the cohort correlated closely with those previously reported in PDAC cell lines and xenografts (Supplementary Table 7).

Significant mutated gene analysis[12] of genes with non-silent mutations that occurred in 2 or more individual cancers identified 16 genes in the top 20 mutated genes in 2 of 3 stringent analytical approaches (Table 2, Supplementary Table 8 and Supplementary Methods) and reaffirmed the importance of mutations known to occur in PDAC: *KRAS, TP53, CDKN2A, SMAD4, MLL3, TGFBR2, ARID1A* and *SF3B1*. Novel significantly mutated genes included additional genes involved in chromatin modification (*EPC1* and *ARID2*) and *ATM*, recently implicated as a PDAC susceptibility gene through bi-allelic inactivation in a case of familial PDAC (germline mutation and loss of heterozygosity (LOH) in the tumour)[13]. Aberrations of *ATM* occurred in 8% of our cohort (mutated in 5%, LOH or loss in 5%, with two patients exhibiting both mutation and LOH or loss) and mutations detected in other genes not previously reported: *ZIM2, MAP2K4, NALCN, SLC16A4* and *MAGEA6* (Table 2). GISTIC2.0[14] identified 30 genes affected by copy-number alterations (Q value <0.0001) and included losses of *CDKN2A* and *SMAD4* (Supplementary Table 4).

Pathways in pancreatic cancer

To better understand potential underlying mechanisms of importance in PDAC, we performed a series of pathway analyses using genes that were recurrently mutated in two or more individuals using GeneGO[15], and identified mechanisms known to be importantin cancer: G1/S checkpoint machinery ($P = 1.49 \times 10^{-3}$), apoptosis ($P = 1.32 \times 10^{-4}$), regulation of angiogenesis ($P = 7.72 \times 10^{-4}$) and TGF-β signalling ($P = 9.50 \times 10^{-4}$). Interestingly, novel gene signatures were enriched in our cohort, including axon guidance ($P = 5.30 \times 10^{-5}$) (Supplementary Table 9). The inclusion of mutation data for 24 cases from ref. 4 strengthened the association of axon guidance ($P = 3.3 \times 10^{-7}$), and was more evident still when all mutated genes in our data set were used as input ($P = 4.67 \times 10^{-8}$).

Functional relevance of genomic events

Differentiating somatic driving events of carcinogenesis from passenger mutations is a major challenge in cancer genomics[16]. Despite significant advances in computational algorithms, experimental evidence of functional relevance is paramount. We used data from three published experimental biological screens to infer functional consequences for the individual genomic events and the pathways we identified. These included data from two independent Sleeping Beauty transposon (SB) mutagenesis screens in *Kras* transgenic mouse models of PDAC[17,18] and an *in vitro* short hairpin RNA (shRNA) screen which examined the consequences of downregulating 11,194 putative cancer genes on survival in a panel of 102 cell lines (13 pancreatic)[19] (Supplementary Methods and Supplementary Figs 5 and 6). Data from these screens confirmed the functional importance of *KRAS, TP53, CDKN2A* and *SMAD4* mutations and attributed potential functional relevance to most significantly mutated genes—MLL3, TGFBR2, SF3B1, EPC1, ARID1A, ARID2, MAP2K4, ATM, NALCN, ZIM2, SLC16A4 (Table 2)—and many genes mutated at low frequency (Supplementary Table 4).

Pathway analysis of high confidence insertions in SB transposon mutagenesis screens demonstrated enrichment for axon guidance genes ($P = 1.6 \times 10^{-3}$), providing independent supportive evidence for a potential role in the pathogenesis of PDAC. In these screens, 14 genes involved in axon guidance pathways were detected (5 genes common to both). In addition, a further 32 genes were mutated in at least one SB pancreatic tumour (out of 21) but did not meet the significance threshold with the stringent analyses that were applied[17] (Supplementary Tables 10 and 11).

Axon guidance pathway genes

The class of genes traditionally described for their roles in axon guidance (semaphorins, slits, netrins and ephrins) are important regulators of normal neuronal migration and positioning during embryonic development. More recently, they have been implicated in cancer cell growth, survival, invasion and angiogenesis[20]; however, the incidence of aberrations in these genes in cancer is largely unknown. We identified recurrent mutations and copy-number variations (CNVs) of axon guidance pathway genes in this cohort (Fig. 1

and Supplementary Table 4): *SLIT2* and *ROBO2* mutations were present in 5% of patients, with focal copy-number losses of *ROBO1*, and *SLIT2* detected by GISTIC2.0 analysis and confirmed by manual review, potentially having an impact on a further 15% of the cohort, suggesting that aberrant SLIT/ROBO signalling is potentially a common feature of PDAC (Figs 1 and 2). In addition, we used targeted PCR-based sequencing of an additional 30 cases of PDAC for axon guidance genes and identified mutations in *ROBO1* in two patients and additional mutations in *SLIT2* and *ROBO2* (one patient each). Low mRNA expression of the *ROBO2* receptor was associated with poor patient survival ($P = 0.04$). Furthermore, high mRNA expression of *ROBO3*, a known inhibitor of *ROBO2* signalling[21], demonstrated an appropriate reciprocal inverse association with poor survival ($P < 0.006$) (Fig. 2).

Class 3 semaphorins (*SEMA3A* and *SEMA3E*) exhibited significant amplification in 18% of patients and an additional 3% harboured mutations (Fig. 1). Semaphorins signal through neuropilin and plexin receptors to elicit their effects[22]. *SEMA3A* amplification correlated with high mRNA expression on microarray ($P = 0.03$), and high mRNA expression of *SEMA3A* and *PLXNA1*, another molecule central to semaphorin signalling, were both associated with poor patient survival on univariate analysis (Fig. 3a), and were independently prognostic on multivariate analyses with clinico-pathological variables (Supplementary Table 12).

To elucidate further the significance of the observed CNV events, we reviewed copy number, CNV segment size and changes in heterozygosity of axon guidance genes in a recent independent CNV analysis of 39 fine-needle aspiration biopsies[23] and the 16 PDAC cell lines in the CONAN database (http://www.sanger.ac.uk/cosmic)[24]. Overall, the predominant changes recapitulated our studies, showing frequent focal losses within genes involved in SLIT/ROBO signalling, and gains in genes involved in canonical semaphorin signalling (Supplementary Tables 4, 13 and 14).

To assess whether dysregulation of axon guidance genes is associated with early neoplastic transformation, as are many developmental signalling pathways, we examined mRNA expression in murine models of early pancreatic carcinogenesis (*in vitro* acinar-to-ductal metaplasia and *in vivo* pancreatic injury). Expression levels of components of SLIT/ROBO and semaphorin signalling changed progressively from normal pancreas, through acinar-to-ductal metaplasia and pancreatic injury to genetically engineered murine PDAC, indicating a role for the dysregulation of these axon guidance genes in tumour initiation and progression (Fig. 3b and Supplementary Table 15).

Discussion

We devised methodologies to optimize mutation detection for clinical samples in a large cohort of patients and reaffirm known mutations in PDAC, better define their prevalence in a large cohort of early PDAC, and identify potential novel drivers in this disease. Somatic mutations in *ATM* were identified in a significant proportion of patients (8%), highlighting the importance of BRCA-mediated DNA damage repair mechanisms in sporadic PDAC as well as familial disease[13]. Previously, mutations in individual genes involved in chromatin remodelling such as *ARID1A*[25] have been described and additional genes identified here (*EPC1* and *ARID2*) infer that chromatin remodelling may have an important role in PDAC, along with other cancer types[26].

Novel mutations in genes traditionally described for their roles in axon guidance were also observed by a combination of genomic data and supportive experimental evidence from independent murine SB mutagenesis screens. Axon guidance is integral to organogenesis, regeneration, wound healing and other basic cellular processes[22,27]. The widespread genomic aberrations observed here in axon guidance genes suggests that they may have a role in PDAC, joining mounting evidence in other cancers[20,28], including a recent report demonstrating *ROBO2* mutations in liver-fluke-associated cholangiocarcinoma[29]. In addition, evidence from cancers of the lung, breast, kidney and cervix implicate aberrant SLIT/ROBO signalling in carcinogenesis[20]; *Robo1* knockout mice develop bronchial hyperplasia and focal dysplasia, and inactivation of *Slit2* and *Slit3* leads to the development of hyperplastic disorganized lesions in the breast[20]. Upregulation of MET and WNT signalling have important roles in PDAC, and recent data indicate that SLIT/ROBO signalling modulates MET and WNT signalling activity through CDC42 and β-catenin, respectively[20]. Loss of SLIT/ROBO signalling can potentially be an alternative mechanism for deregulating these pathways downstream of their receptors, and in addition could influence the activity of inhibitors that target these upstream components, for example, MET inhibitors (Fig. 2).

Class 3 semaphorins are the only secreted semaphorins in vertebrates. They regulate cell growth, invasiveness and angiogenesis, and are highly expressed in metastatic cells in many cancer types[30,31]. Although aberrant semaphorin signalling in cancer seems to be organ specific[32], our finding that high expression of *SEMA3A* and its receptor *PLXNA1* co-segregates with poor patient survival is supported by a previous study that reported this association and also demonstrated promotion of invasiveness of PDAC cell lines by *SEMA3A*[31]. Therapeutics targeting molecules involved in axon guidance have been developed as potential strategies to facilitate neuronal regeneration after injury[33], but are yet to be assessed for their role in cancer treatment.

As illustrated here, global genomic analysis of large, well-annotated and clinically homogeneous cohorts of patients can identify mechanisms that are common among genomically diverse cancers, and will be pivotal in the development of novel therapeutic strategies that are guided by the determination of the molecular phenotype of individual patients[34]. Future work will be required to determine which key components, when damaged, drive the disease, and these mechanisms will need to be assessed in molecularly well-characterized preclinical models[35]. The potential therapeutic strategies identified will then require testing in appropriate clinical trials that are specifically designed to target subsets of patients stratified according to well-defined molecular markers[36,37].

METHODS SUMMARY

Sample acquisition and processing

Samples used were prospectively acquired and restricted to primary operable, non-pretreated pancreatic ductal adenocarcinoma. Representative sections were reviewed independently by at least one additional pathologist with specific expertise in pancreatic diseases. Samples either had full face frozen sectioning performed in optimal cutting temperature (OCT) medium, or the ends excised and processed in formalin to verify the presence of carcinoma in the sample to be sequenced and to estimate the percentage of malignant epithelial nuclei in the sample relative to stromal nuclei. Macrodissection was performed if required to excise areas that did not contain malignant epithelium.

Sequencing

Cellularity of each tumour sample was estimated with pathology review, deep sequencing of *KRAS* and a method developed using genome-wide SNP array data (qpure[10]). Exon capture was performed using the SureSelect II or Nimblegen capture methods and paired-end sequenced on the SOLiD (v4) or GAII/HiSeq platforms. Somatic mutations were called and then verified on the Ion Torrent Personal Genome Machine (Life Technologies Corporation) and 454 (Hoffman–La Roche Limited).

Analysis

Significantly mutated genes were identified using the Genome MuSiC package[12]. DNA copy number analyses were performed using the Illumina HumanOmni1 Quad genotyping arrays and GenoCN software. Recurrent and significant copy number changes were identified using GISTIC2.0[14]. Functional enrichment of gene categories was assessed using the Metacore package (Thomson-Reuters Corporation) and the MSigDB v3.0 database[38]. All sample information and data for mutation, copy number and expression analyses were submitted to the ICGC DCC at http://dcc.icgc.org/. A complete description of the materials and methods including approvals for human research and animal experimentation is provided in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Australian Pancreatic Cancer Genome Initiative

The Kinghorn Cancer Centre, Garvan Institute of Medical Research Andrew V. Biankin[1], Amber L. Johns[1], Amanda Mawson[1], David K. Chang[1], Christopher J. Scarlett[1], Mary-Anne L. Brancato[1], Sarah J. Rowe[1], Skye L. Simpson[1], Mona Martyn-Smith[1], Michelle T. Thomas[1], Lorraine A. Chantrill[1], Venessa T. Chin[1], Angela Chou[1], Mark J. Cowley[1], Jeremy L. Humphris[1], Marc D. Jones[1], R. Scott Mead[1], Adnan M. Nagrial[1], Marina Pajic[1], Jessica Pettit[1], Mark Pinese[1], Ilse Rooman[1], Jianmin Wu[1], Jiang Tao[1], Renee DiPietro[1], Clare Watson[1], Rachel Wong[1], Andreia V. Pinho[1], Marc Giry-Laterriere[1], Roger J. Daly[1], Elizabeth A. Musgrove[1], Robert L. Sutherland[1]; Queensland Center for Medical Genomics, Institute for Molecular Bioscience Sean M. Grimmond[2], Nicola Waddell[2], Karin S.

Kassahn[2], David K. Miller[2], Peter J. Wilson[2], Ann-Marie Patch[2], Sarah Song[2], Ivon Harliwong[2], Senel Idrisoglu[2], Craig Nourse[2], Ehsan Nourbakhsh[2], Suzanne Manning[2], Shivangi Wani[2], Milena Gongora[2], Matthew Anderson[2], Oliver Holmes[2], Conrad Leonard[2], Darrin Taylor[2], Scott Wood[2], Qinying Xu[2], Katia Nones[2], J. Lynn Fink[2], Angelika Christ[2], Tim Bruxner[2], Nicole Cloonan[2], Felicity Newell[2], John V. Pearson[2]; **Royal North Shore Hospital** Jaswinder S. Samra[3], Anthony J. Gill[3], Nick Pavlakis[3], Alex Guminski[3], Christopher Toon[3]; **Bankstown Hospital** Andrew V. Biankin[4], Ray Asghari[4], Neil D. Merrett[4], David K. Chang[4], Darren A. Pavey[4], Amitabha Das[4]; **Liverpool Hospital** Peter H. Cosman[5], Kasim Ismail[5], Chelsie O'Connor[5]; **Westmead Hospital** Vincent W. Lam[6], Duncan McLeod[6], Henry C. Pleass[6], Arthur Richardson[6], Virginia James[6]; **Royal Prince Alfred Hospital** James G. Kench[7], Caroline L. Cooper[7], David Joseph[7], Charbel Sandroussi[7], Michael Crawford[7], James Gallagher[7]; **Fremantle Hospital** Michael Texler[8], Cindy Forrest[8], Andrew Laycock[8], Krishna P. Epari[8], Mo Ballal[8], David R. Fletcher[8], Sanjay Mukhedkar[8]; **Sir Charles Gairdner Hospital** Nigel A. Spry[9], Bastiaan DeBoer[9], Ming Chai[9]; **St John of God Healthcare** Nikolajs Zeps[10], Maria Beilin[10], Kynan Feeney[10]; **Royal Adelaide Hospital** Nam Q. Nguyen[11], Andrew R. Ruszkiewicz[11], Chris Worthley[11], Chuan P. Tan[11], Tamara Debrencini[11]; **Flinders Medical Centre** John Chen[12], Mark E. Brooke-Smith[12], Virginia Papangelis[12]; **Greenslopes Private Hospital** Henry Tang[13], Andrew P. Barbour[13]; **Envoi Pathology** Andrew D. Clouston[14], Patrick Martin[14]; **Princess Alexandria Hospital** Thomas J. O'Rourke[15], Amy Chiang[15], Jonathan W. Fawcett[15], Kellee Slater[15], Shinn Yeung[15], Michael Hatzifotis[15], Peter Hodgkinson[15]; **Austin Hospital** Christopher Christophi[16], Mehrdad Nikfarjam[16], Angela Mountain[16]; Victorian Cancer Biobank[17]; **Johns Hopkins Medical Institutes** James R. Eshleman[18], Ralph H. Hruban[18], Anirban Maitra[18], Christine A. Iacobuzio-Donahue[18], Richard D. Schulick[18], Christopher L. Wolfgang[18], Richard A. Morgan[18], Mary B. Hodgin[18]; **ARC-NET Center for Applied Research on Cancer** Aldo Scarpa[19], Rita T. Lawlor[19], Paola Capelli[19], Stefania Beghelli[19], Vincenzo Corbo[19], Maria Scardoni[19], Paolo Pederzoli[19], Giampaolo Tortora[19], Claudio Bassi[19]; **University of California, San Francisco** Margaret A. Tempero[20]

[1]The Kinghorn Cancer Centre, Cancer Research Program, Garvan Institute of Medical Research, 370 Victoria Street, Darlinghurst, Sydney, New South Wales 2010, Australia. [2]Queensland Center for Medical Genomics, Institute for Molecular Bioscience, University of Queensland, St Lucia, Queensland 4072, Australia. [3]Royal North Shore Hospital, Westbourne Street, St Leonards, New South Wales 2065, Australia. [4]Bankstown Hospital, Eldridge Road, Bankstown, New South Wales 2200, Australia. [5]Liverpool Hospital, Elizabeth Street, Liverpool, New South Wales 2170, Australia. [6]Westmead Hospital, Hawkesbury and Darcy Roads, Westmead, New South Wales 2145, Australia. [7]Royal Prince Alfred Hospital, Missenden Road, Camperdown, New South Wales 2050, Australia. [8]Fremantle Hospital, Alma Street, Fremantle, Western Australia 6959, Australia. [9]Sir Charles Gairdner Hospital, Hospital Avenue, Nedlands, Western Australia 6009, Australia. [10]St John of God Healthcare, 12 Salvado Road, Subiaco, Western Australia 6008, Australia. [11]Royal Adelaide Hospital, North Terrace, Adelaide, South Australia 5000, Australia. [12]Flinders Medical Centre, Flinders Drive, Bedford Park, South Australia 5042, Australia. [13]Greenslopes Private Hospital, Newdegate Street, Greenslopes, Queensland 4120, Australia. [14]Envoi Pathology, 1/49 Butterfield Street, Herston, Queensland 4006, Australia. [15]Princess Alexandria Hospital, Cornwall Street & Ipswich Road, Woolloongabba, Queensland 4102, Australia. [16]Austin Hospital, 145 Studley Road, Heidelberg, Victoria 3084, Australia. [17]Victorian Cancer Biobank, 1 Rathdowne Street, Carlton, Victoria 3053, Australia. [18]Johns Hopkins Medical Institutes, 600 North Wolfe Street, Baltimore, Maryland 21287, USA. [19]ARC-NET Center for Applied Research on Cancer, University of Verona, Via dell'Artigliere, 19 37129 Verona 37134,

Italy. [20]University of California, San Francisco, 1600 Divisadero Street, San Francisco, California 94115, USA.

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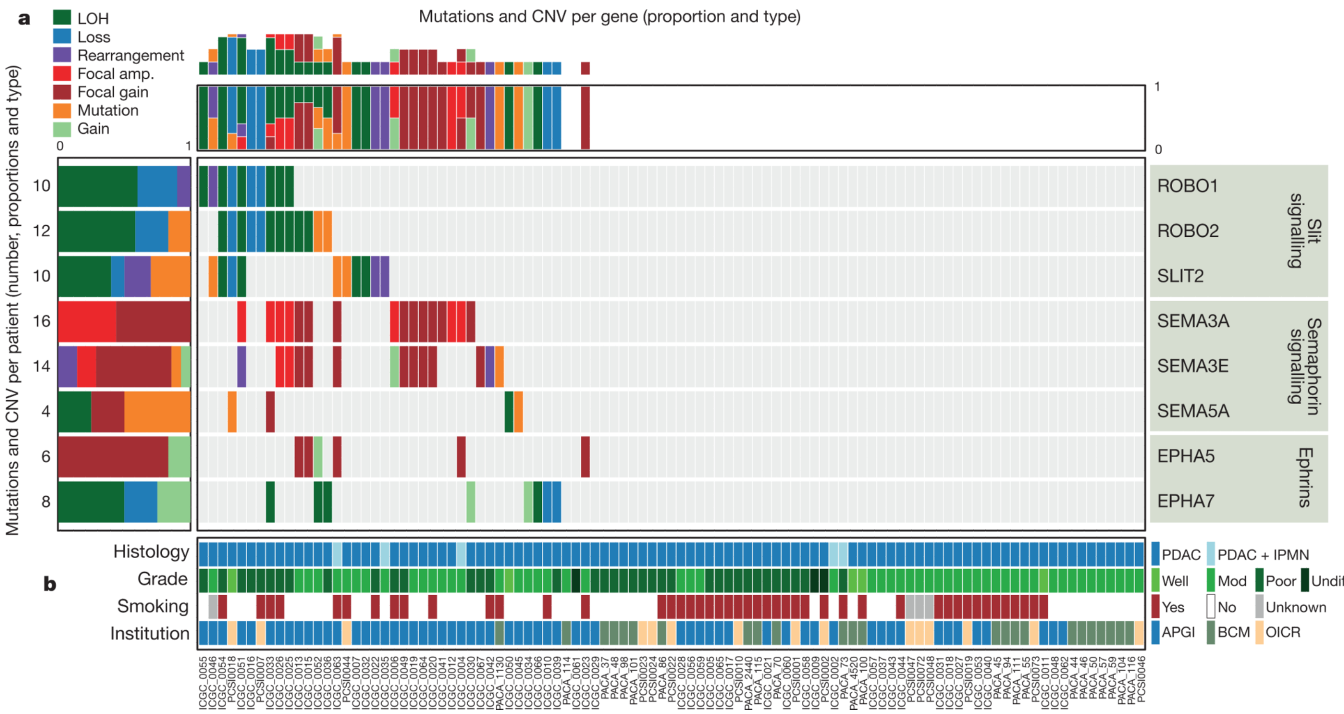

Figure 1. Mutations and copy number variation in axon guidance genes

Axon guidance pathway genes with recurrent mutations and/or copy-number changes defined by GISTIC2.0 analysis ($Q < 0.2$), and manually reviewed for focal alterations. **a**, SNV and CNV frequency per patient with gene-centric summary (left) and patient-centric summary (top); numbers of patients with mutations and proportion of each event are presented. Please see Supplementary Table 4 for further details. **b**, Clinico-pathological variables for individual patients. APGI, Australian Pancreatic Cancer Genome Initiative; BCM, Baylor College of Medicine; IPMN, intraductal papillary mucinous neoplasm; Mod, moderately differentiated; OICR, Ontario Institute for Cancer Research; PDAC, pancreatic ductal adenocarcinoma; Undiff, undifferentiated.

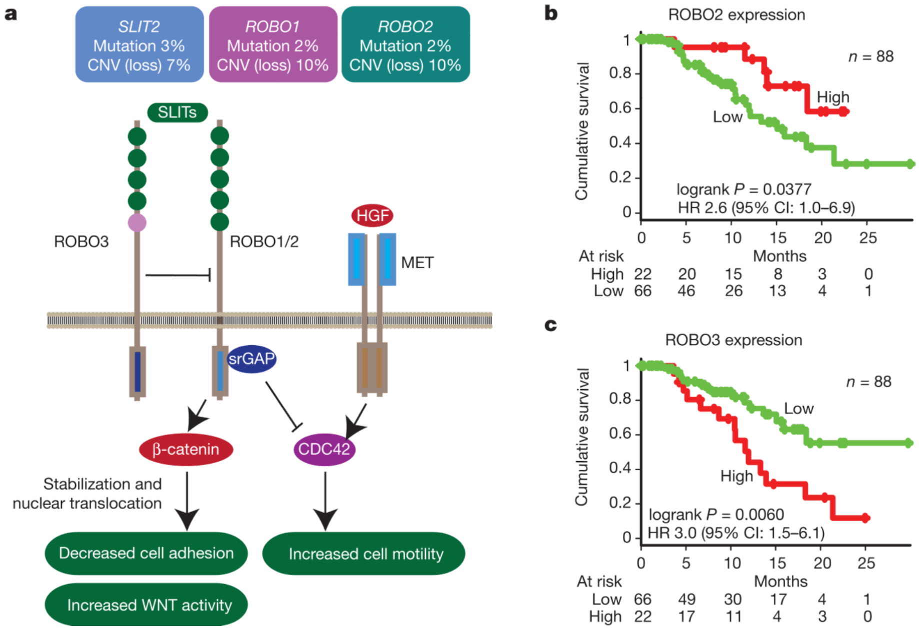

Figure 2. SLIT/ROBO signalling in pancreatic ductal adenocarcinoma

a, SLIT/ROBO signalling normally enhances β-catenin complex formation with E-cadherin and suppresses WNT signalling activity. Loss of ROBO1/2 signalling promotes stabilization of β-catenin, which decreases E-cadherin complex formation and cell adhesion and augments WNT signalling activity through increased nuclear translocation of β-catenin. In addition, SLIT/ROBO signalling can downregulate MET signalling activity; loss of ROBO signalling activity promotes MET signalling downstream and may have an impact on therapeutic strategies aimed at inhibiting MET activity at the receptor level. (Adapted from ref. 20.) Aberrations in *SLIT2* and/or *ROBO1/2* affected 23% of patients (6% mutated with 1 patient showing mutations in both *SLIT2* and *ROBO2*), with 18% demonstrating CNV corresponding to loss of the gene. **b, c**, High expression of SLIT receptor ROBO2 was associated with a better prognosis (**b**), and high expression of ROBO3, an inhibitor of ROBO2, showed an inverse relationship, with high levels associated with poor survival (**c**). HR, hazard ratio.

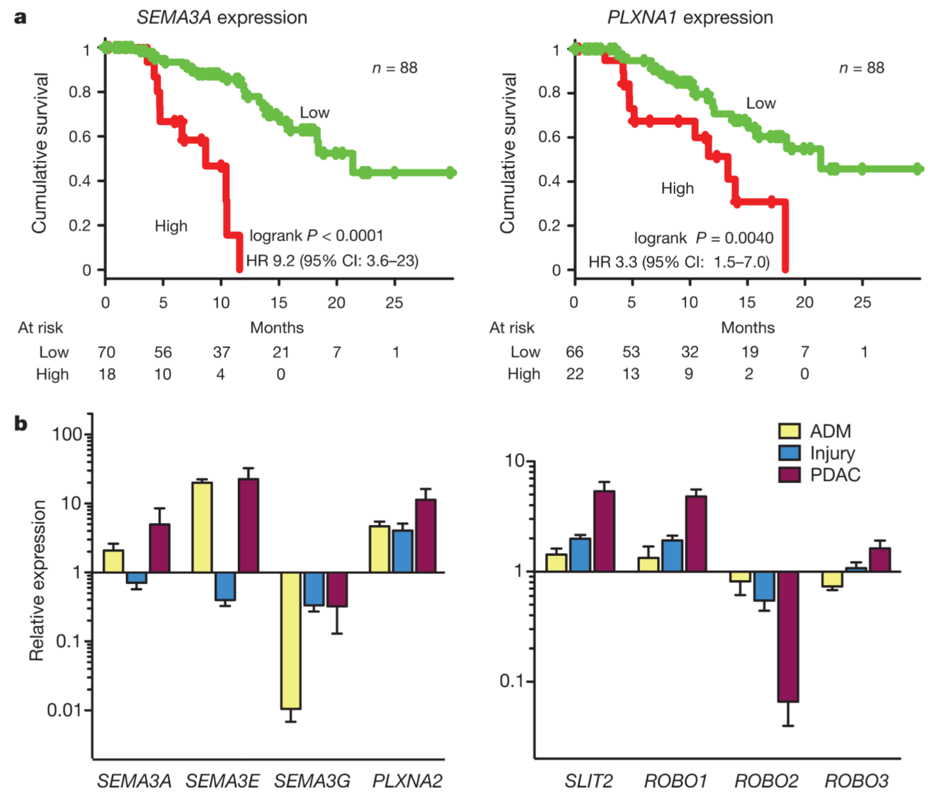

Figure 3. Axon guidance genes in human and murine pancreatic ductal adenocarcinoma

a, Kaplan–Meier survival curves showing co-segregation of aberrant expression of components of semaphorin signalling with outcome. Amplification at *SEMA3A* and *PLXNA1* loci was associated with high mRNA expression and both are independent poor prognostic factors. **b**, Quantitative RT–PCR for components of semaphorin and SLIT/ROBO signalling in murine models of early (acinar-to-ductal metaplasia (ADM) and pancreatic injury) and established PDAC in genetically engineered mice with a *Pdx1*-promoter-driven activating mutation of *Kras* and mutant *Tp53* allele (*Pdx1-Cre; LSL-*$Kras^{G12D}$*; LSL-*$Trp53^{R172H}$). Error bars represent standard error of the mean (see Supplementary Table 15 for details).

Table 1

Mutations in pancreatic ductal adenocarcinoma (*n* = 99)

Mutation class	Total
Missense	1,684
Nonsense	99
Splice site	89
Insertion/deletion	144
Non-silent	2,016
Silent	611

Table 2

Significantly mutated genes in pancreatic ductal adenocarcinoma

Gene symbol	Gene name and protein function	SB mutagenesis*	shRNA†
KRAS	Oncogene; GTPase; activation of MAPK activity	Yes	Yes
TP53	Tumour suppressor p53; DNA damage response	–	Yes
CDKN2A	Cyclin-dependent kinase inhibitor 2A; G1/S transition of mitotic cell cycle; tumour suppressor	Yes	–
SMAD4	Mothers against decapentaplegic homologue 4; BMP signalling pathway	Yes	Yes
MLL3	Myeloid/lymphoid or mixed-lineage leukaemia protein 3; DNA binding; regulation of transcription	Yes	Yes
TGFBR2	Transforming growth factor-β receptor type II; regulation of growth	Yes	–
ARID1A	AT-rich interactive domain-containing protein 1A; SWI/SNF complex; chromatin modification	Yes	Yes
ARID2	AT-rich interactive domain-containing protein 2; chromatin modification	Yes	–
EPC1	Enhancer of polycomb homologue 1; histone acetylation	Yes	–
ATM	Ataxia telangiectasia mutated; DNA damage response	–	Yes
SF3B1	Splicing factor 3B subunit 1; nuclear mRNA splicing	–	Yes
ZIM2	Zinc finger imprinted 2; regulation of transcription	–	Yes
MAP2K4	Dual specificity mitogen-activated protein kinase kinase 4; Toll-like receptor signalling pathway	Yes	Yes
NALCN	Sodium leak channel non-selective protein; sodium channel activity	–	Yes
SLC16A4	Solute carrier family 16 member 4; monocarboxylate transporter	–	Yes
MAGEA6	Melanoma-associated antigen 6; protein binding	–	ND

ND, not determined.

*Significant insertion sites in two independent Sleeping Beauty mutagenesis screens[17,18].

†*In vitro* shRNA screens in 102 cancer cell lines with effect on cell survival[19].