Laser Capture Microdissection

Subjects: Biochemistry & Molecular Biology

Contributor: Bhavana Hemantha Rao , Pavel Souček , Viktor Hlaváč

The advancement in molecular techniques has been attributed to the quality and significance of cancer research. Pancreatic cancer (PC) is one of the rare cancers with aggressive behavior and a high mortality rate. The asymptomatic nature of the disease until its advanced stage has resulted in late diagnosis as well as poor prognosis. The heterogeneous character of PC has complicated cancer development and progression studies. The analysis of bulk tissues of the disease was insufficient to understand the disease, hence, the introduction of the single-cell separating technique aided researchers to decipher more about the specific cell population of tumors.

Laser Capture Microdissection (LCM) pancreatic cancer intraductal papillary mucinous neoplasm (IPMN) single-cell separation

1. Laser Capture Microdissection (LCM)

LCM is a sophisticated technique in which a laser is coupled with an inverted microscope and linked to a computer. This technique was developed in 1996 at the National Institute of Cancer, USA, for isolating selected human cell populations from a heterogeneous population of cells [1]. The laser systems used in this apparatus have been modified since then from ultraviolet (UV) to high-energy nitrogen, infrared, and carbon dioxide lasers [2]. Based on the laser beams used, LCM can be of two types: infrared and UV. The commercially available Arcturus Pixcell IIE LCM platform is an example of infrared LCM, whereas the PALM Laser-MicroBeam System, MMI cellcut® (Molecular Machines and Industries), and the Leica Laser Microdissection system, are commercialized instruments in which UV-laser beams are installed [2][3][4][5]. The latter is a widely used instrument in which the solid tissues are typically prepared on a membrane-covered slide to identify the target cells and examined under the microscope [1], whereas the cell culture specimens are cultured on membrane-bound culture plates to which the cells adhere [6]. The membranes are usually polyethylene naphthalate, to which methods such as hematoxylin/eosin staining, fluorescent in situ hybridization (FISH), and immunohistochemistry (IHC) are used for staining the cells [2]. Then, the cells of interest are manually marked on the computer screen, and the laser cuts along the marked direction [1]. This is followed by contact-based extraction, contact-free gravity-assisted microdissection (GAM), or contact-free laser pressure catapulting (LPC), after which the cells are treated with appropriate buffers for processing and sequencing [7]. While working with the frozen tissue sample, careful handling during the cryosectioning, staining, and marking of the cells of interest for microdissection aids in yielding a high-quality nucleic acid for next-generation sequencing [8].

Like every technique, LCM has benefits and drawbacks. It enables small tissue isolation from a heterogeneous population in a single step through direct visualization through a microscope; it is a fairly quick method of dissection; it secures the tissue morphology during the dissection; and it also allows to separate live cells/single cells in a culture dish and re-culture them. Especially when the content of tumor cells in neoplastic tissue is low, LCM takes the advantage of enriching the tumor fraction. The drawbacks of this method include its high cost, the need for a histologist or other specialized personnel to identify the cells of interest, and the possibility that the quality of the dissected tissue will not meet the standards needed for the further processing of the sample because of the absence of a coverslip, which causes dehydration of the sample [2][5]. When compared to FACS and microfluidics systems, which are often used on liquid samples especially for separating cells from the blood, LCM can isolate the single cells from tissue samples like FFPE and fresh frozen, whereas the need for professional personnel to identify the cells holds the disadvantage of LCM. In addition, though it is time-consuming, the tumor cells can be isolated using a laser beam directly without treating the samples with fluorophores, which is often done in both FACS and microfluidics [9][10]. Having advantages and disadvantages of the technique, LCM is an efficient tool for isolating single cells from tissue samples.

The application of LCM in cancer research was reviewed in some solid cancers. Lawrie and Curran [11] described the use of LCM in colorectal cancer proteomics; Fuller et al. [3] reviewed the utility of LCM in breast cancer; neuroblastoma [12] and prostate cancer [13] were also reviewed. However, this was before the onset of novel techniques such as next-generation sequencing. Some recent reviews advocate the use of LCM in oral cancer [14] or testicular germ cell tumors [15]. Liotta et al. [16] described the use of LCM in the protein analysis of solid cancers and methodology, with example applications in cancer tissues, thoroughly reviewed in von Eggeling and Hoffmann [17]. However, the whole omics view on the use of LCM in PC is completely missing.

2. Mutation Studies

The heterogeneous nature of PDAC is characterized by various genetic alterations like the activation of the proto-oncogene, *KRAS*; the inactivation of *CDKN2A*, *TP53*, *SMAD4*, and *STK11*/LKB1, the tumor suppressor genes [18]; the loss of heterozygosity at 19p13.3 [19], 6q and 17p (in IMPNs) [20], and so on. These alterations are thoroughly studied to understand their role in PC metastasis, seek their role in prognosis/survival, or identify therapeutic targets [19]. Before the use of single-cell separation methods, the bulk tissues were analyzed for the research in PC. The mutations of *KRAS* and *TP53*, inactivation of p16/*CDKN2A*, and *SMAD4*/DPC4 in PanIN, IPMN, and MCN were found in the analysis of FFPE samples of tumor tissues [21].

KRAS mutation was found to be an early event in all three precancerous lesions accompanied by p16/*CDKN2A* inactivation in PanIN and IPMN. Whereas *TP53* mutation and silencing of *SMAD*/DPC4 were the late events in PanIN, IPMN, and MCN [21]. Also, the frequent mutation of *KRAS* at codon 12 and exceptionally at codon 13, and 61; the difference in the pattern of *KRAS* mutation in Japanese and European populations with GGT to GAT (G12D) in Japanese and GGT to GAT (G12D), GGT to GTT (G12V), CGT (G12R) or TGT (G12C) in European population respectively were discovered before the introduction of LCM [21].

Using LCM, most of these findings were confirmed like the typical *KRAS*, *SMAD*/*DPC4*, and *TP53* mutations, along with the somatic mutation of *PIK3CA* in MCN [22], and along with it, many more interesting facts were deduced from the specific cell population of the tumors such as the mutation analysis conducted by Crnogorac-jurcevic et al. two decades ago using LCM treated normal and tumor samples of PDAC revealing the homozygous deletion of *CDKN2* and the mutation of *KRAS* using single-strand conformation polymorphism (SSCP) and direct sequencing methods. In the same experiment, they used a cDNA array, tissue array, and IHC to discover the involvement of overexpressed genes *TIMP1*, *CD59*, *ABL2*, *NOTCH4*, *SOD1*, and the downregulation of *XRCC1* gene in different pathways leading to pancreatic malignancy [23].

Similarly, the population-based study conducted on the Japanese and European populations was performed on Chinese populations using LCM, PCR, and direct sequencing. The *KRAS* mutations in the Chinese population were found to be different compared to the Japanese-European population with a mutation in the first or second base of the codon 12 (GGT) [24]. A similar study on *KRAS* and *TP53* gene mutation in PDAC patients from highly polluted regions of the Nile River delta in Egypt to the less polluted region showed a significantly higher rate of mutation in the *KRAS* codon 12 G to T (G12V) transversion mutation and mutation in exon 5-8 of TP53 in patients from highly polluted areas, pointed out the importance of the interaction of environment and genes in carcinogenesis [25].

A different study was carried out by Izawa et al. [26] on LCM-derived IPMN tissues to study the clonal characterization using the combination of *KRAS* analysis and analysis of human androgen receptor gene (HUMARA) during X-chromosome inactivation. The study concluded the polyclonal/oligoclonal nature of IPMNs and their origin from multiple precancerous lesions. Pancreas is made up of different cells like ductal, stellate, acinar, and beta cells among which, acinar cells perform the role of secreting digestive enzymes namely amylase, protease, and lipase in the form of zymogens [27].

The analysis of the whole-tumor tissue had given researchers the idea that acinar cells could be the origin of human pancreatic neoplasia but with the help of LCM, PanIN lesions, acinar-ductal metaplasia lesions, stromal cells, and acinar cells were isolated and closely studied for *KRAS* mutation (LigAmp technique) to disapprove this hypothesis [28]. There were contradictory findings like the study using LCM showing the *TP53* gene could be found in the early stage of PDAC, which was found to be a late-stage event by the study on bulk tissue, but the same experiment supported that *KRAS* mutation along with other somatic gene mutations found in early-stage PanIN-2 lesions promotes the PDAC progression [29]. However, a recent study substantiated the role of *TP53* in the evolution of PDAC and found that *TP53* is not only a gateway to genetic chaos but also a provider of deterministic patterns of genome evolution that may show new strategies for the treatment of tumors with *TP53* mutation [30].

Carcinosarcoma is also one among the rare PC, and the study performed by Bai et al. [31] with the help of LCM to isolate carcinomatous and sarcomatous cells from carcinosarcoma samples, were studied for IHC, clinicopathological, and *KRAS* mutation, which showed similar mutation pattern in *KRAS* mutation (p.G12D and p.G12V) in both the samples, indicating that both the components has a monoclonal origin.

The conflict in the results from bulk and specific cell population also includes the study of epidermal growth factor (*EGFR*) that plays a critical role in many cancer types, with its downstream pathways including RAS-MAPK/PI3K-AKT-mTOR pathways that are well studied in cancer prognosis [32]. However, the study on pancreatic cell lines and clinical samples using LCM and direct sequencing revealed that the *EGFR* gene is highly conserved in pancreatic cancer and contradicted its association with PC prognosis, leaving room for other explanations of the relevance of *EGFR* mutation in PDAC [33]. Additionally, one of the simulation studies performed by Fujii et al. [34] demonstrated the lack of microsatellite instability in PC using specialized fluorescent microsatellite analysis on microdissected PDAC specimens, but the study showed profound LOH in these samples. Further, they recommended against the usage of LCM in microsatellite instability studies using tissue samples.

Several other fascinating observations about *KRAS*, such as the study demonstrating the carcinogenic role of the secretory and trophic effects-regulating hormone, gastrin, was carried out on gastrin gene-knockout, *KRAS*-mutant mice, and in human samples microdissected using LCM. The results were interesting as the knockout mutant mice showed decreased PanIN progression, inflammation, and fibrosis compared to the results obtained from the re-expression of gastrin. The decrease in KRAS expression reverted the signal transduction to the canonical pathway and they found a significant increase in the gastrin mRNA expression in PC samples when it was re-expressed. Hence, with the help of LCM on healthy pancreatic tissue and tumor tissues, the expression study of gastrin unveiled its possible role in activating *KRAS* in PC [35].

3. Breakthrough of PC Subtypes and Their Relevance in Survival

The integration of genomics, transcriptomics, proteomics, methylation studies, and other omics studies, can help better understand and identify biomarkers of early diagnosis, prognosis, or therapy prediction of cancer patients. It also helps to identify the targets for treatment. The study on molecular subtypes of pancreatic cancer contributed to understanding the survival of patients. Collisson et al. used LCM-based techniques to distinguish cancer and stromal subtypes of PC [36]. Thereafter, Moffitt et al. [37] determined the PC subtypes using an algorithm-based virtual microdissection on PDAC tissue samples and validated the use of bulk RNA-sequencing data using the Non-negative matrix factorization (NMF) method. A similar study was conducted by Kalloger et al. [38].

Recently, Birnbaum et al. [4] took an effort to conduct a transcriptomic study to explore the role of PC subtypes of cancer and stromal cells in prognosis and precision medicine. They identified four cancer subtypes (C1–C4) and three stromal subtypes (S1–S3) and they correlated it with the short-term survival and long-term survival using differentially expressed gene (DEG) analysis. The canonical pathway and Gene Ontology (GO) biological process evaluated the involvement of the C1 subtype in protein folding and leukocyte chemotaxis; C2 in neuronal membrane signaling and pancreatic endocrine cell development; C3 in protein translation regulation and nucleotide biosynthesis; C4 in the oncogenic signal transduction pathway; S1 in cell development and differentiation; S2 in antigen processing and presentation; and S3 in macromolecular modification. These sub-types were identical to Bailey, Collison, Moffitt, and Puleo's classifications, in which, C1 and C3 were found similar to classical or

pancreatic progenitor subtypes, C2 to ADEX, or exocrine-like subtype, and C4 to squamous or basal-like or quasi-mesenchymal subtype [4][36][37][39].

From the gene expression study conducted for prognosis and survival, genes associated with short-term survival were associated with cell plasticity, axon guidance, cell proliferation, and signal transduction; whereas long-term survival was associated with cell cycle regulation and tRNA/mRNA processing. Out of 113 genes, 13 genes were found to be exclusively expressed in cancer cells and they were confirmed by the two-color RNA-ISH (RNA-In situ Hybridisation). Genes *AP5M1, TCP1,* and *PNP* associated with long-term- and *MIA*, *MUC16*, and *ADGRF1* associated with short-term survival were highlighted as gene signatures for survival [4]. The microdissection technique was used for the investigations on subtypes and their association with survival within the recent prospective trial study, COMPASS, initiated at the Princess Margaret Cancer Centre in Toronto. Researchers used metastatic tumor cells to study the predictive mutational and transcriptional characteristics of PDAC for better treatment selection [40]. Whole-genome sequencing and RNA-sequencing of the microdissected samples revealed that the subtypes from the III/IV stage of PDAC were similar to Moffitt et al. classical subtype tumors and their response to mFOLFIRINOX first-line chemotherapy was better compared to the basal-like tumors. They also highlighted the importance of *GATA6* expression in differentiating the classical and basal-like subtypes in PDAC [40].

Apart from the cancer subtypes studies, the study conducted by Nakamura et al. [41] on DEG of different zones of same PC samples isolated from mice implanted with Human L3.6pl PC cells analysed using LCM, affymetrix GeneChip hybridisation techniques concluded that it is important to understand expression profiles of zonal heterogeneity in the discovery of prognostic and therapeutic biomarkers, and LCM aids in the reproducibility of the analysis in such studies.

4. Proteins, Pathways, and Cancer Management

Understanding the protein profile of the cancer is always of key importance, as it helps to enlighten the pathways leading to cancer development and metastasis [42]. The proteomic studies could be of two types: expression proteomics and functional proteomics. The studies that focus on the upregulation and down-regulations of proteins are the expression proteomics, whereas the studies that focus on the molecular mechanism and the unraveling of the biological functions of novel proteins are called the functional proteomics study [43]. Like the proteomic studies conducted on bulk tissues, which is not the scope of this discussion, single-cell separation methods, especially LCM, are also employed to understand the expression and functional proteomics of PC.

In PC, there are several proteins such as the S100 family, a small integrin-binding ligand N-linked glycoprotein (SIBLING) family, and secreted protein acidic and rich in cysteine (SPARC) family proteins associated with cancer progression [44][45]. Bone Sialoprotein (BSP) is a member of the SIBLING family of proteins and was studied using LCM along with qPCR, DNA microarray, immunoblotting, radio-immunoassays, IHC, cell-growth, invasion, scattering, and adhesion assays on chronic pancreatitis, PDAC, and PC cell lines, to mark its importance in cancer growth, and metastasis [46].

SPARC-like protein 1 (SPARCL1), a SPARC family protein, aka Hevin, found in the extracellular matrix was studied in LCM-applied normal pancreatic tissue and PDAC samples, using qPCR, and other protein analyzing methods. SPARCL1 expression was elevated in PDAC samples compared to the normal tissue and PC cell lines, and its expression was found to be downregulated in the late stages of PC indicating the role of SPARCL1 as a tumor suppressor gene [47].

Similarly, the S100 family proteins are another widely studied, calcium-binding protein family, which has the potential to contribute to the early detection and prognosis of PC [44]. S100A6 was investigated with the help of LCM by A.R. Shekouh et al. They performed LCM with 2D-gel electrophoresis and other techniques such as isoelectric focusing, silver staining, MALDI-TOF, and IHC on normal and malignant tissue samples and validated that this calcium-dependent protein is highly expressed in tumor cells compared to the normal tissues [48]. The same combination of techniques, along with fluorescence dye saturation labelling, was performed on PanIN and normal samples along with comparing the data with proteome reference to find the role of three actin filament proteins (actin, transgelin, and vimentin) in PC progression [49]. S100P, a member of the same family, along with another protein 14-3-3 sigma/SFN, was found to be a promising biomarker in a study on PDAC and its matched lymph node metastasis FFPE sample microdissected using LCM [50]. Later, a study conducted by F Robin et al. tackled the molecular profile of stroma from fresh frozen PDAC, separated using LCM, and analyzed using genome-wide expression profiling, tissue microarray, IHC, and ELISA to conclude that SFN/14-3-3 sigma/stratifin can be a potential candidate for the prognostic biomarker of PDAC [51]. It was clear that stratifin (14-3-3 sigma) played a vital role in cell cycle regulation and apoptosis using the combination of LCM, qPCR, DNA arrays, IHC, and western blotting [52]. The interesting fact is that these proteins stimulate the downstream main pathways like KRAS, apoptosis, DNA damage control, regulation of G1/S phase transition, Hedgehog, and many more [45][52], which gives them the potential to be used as diagnostic or prognostic biomarkers, as well as possible therapeutic targets.

Chronic pancreatitis (CP) is one of the risk factors for PC, a study comparing the protein expression in LCM performed CP, PDAC, and normal cells adjacent to infiltrating PDAC samples, were studied and deciphered the significant expression of cartilage glycoprotein-39 (HC gp-39), pancreatitis-associated proteins (HIP/PAP), and lactoferrin in both the samples compared to the healthy tissue indicating the potential role of these proteins as a predictive biomarker [53].

The study conducted by Sawai et al. [54], on one of the DNA editing enzyme, activation induced cytidine deaminase (AID), in the microdissected PDAC and normal tissue showed a significant increase in AID expression in acinar ductal metaplasia, PanIN, and PDAC suggesting the involvement of the protein in inducing cancer. It was further validated by deep sequencing the samples obtained from transgenic AID mice.

However, by employing LCM, researchers have made the initial step toward identifying several more proteins associated with PC that has not yet been fully studied [55][56][57][58][59][60][61], like the downregulation of Cav-1 as a possible prognostic marker in PC [57]. They have tried to understand the tumor progression using LCM along with proteomic studies that included LC-MS/MS, tissue microarray, and IHC on fresh frozen PDAC and adjacent normal

tissues [56]. There are also studies using LCM showing the influence of CTCs [58], lncRNA H19 [59], HOTTIP [60], and FN1-ITGA-3 [61] on PC prognosis, which has to be studied in detail for further clarifications.

The hypoxic environment of PC is another widely investigated area. During cancer progression, the cells undergo rapid proliferation resulting in consuming a huge amount of oxygen. The drastic alteration in the oxygen levels stimulates a number of proteins such as Insig2, HIF1A, and BNIP3, which in turn activates the downstream pathways, which leads to more aggressive behavior and therapy resistance in PC [62][63][64][65][66]. Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors made of two subunits, alpha and beta (HIFα and HIFβ) [65]. HIFα is known to induce the *VEGF* (vascular endothelial growth factor), *PDGFA* (platelet-derived growth factor alpha), and *FGF2* (coding basic fibroblast growth factor, bFGF), but has not been explored much [63][66]. *HIF1A* induces the glycolytic enzymes as well, the *PGK1* (phosphoglycerate kinase 1) is one such enzyme found overexpressed in microdissected PDAC samples analyzed using proteomic studies. They also marked its potential to act as a diagnostic biomarker or as a therapeutic target [67]. A study conducted on microdissected FFPE samples of PDAC by qPCR and other statistical analysis showed the correlation between the genes *HIF1A*, *FGF2*, *VEGF*, and *PDGFA* in PC development and the significance of *HIF1A* in prognosis [63]. Inspired by recent research on insulin-induced gene 2 (*INSIG2*) as a novel biomarker for colon cancer, Kayashima et al. attempted to study the involvement of *INSIG2* in pancreatic malignancy. They analyzed *INSIG2* mRNA expression on laser microdissected normal pancreatic epithelial cells, invasive ductal carcinoma cells, and PanIN cells, as well as on PC cell lines cultured under normoxic (21% O_2) and hypoxic (<1% O_2) conditions. They found a significant increase of *INSIG2* expression in the PC cell line under the hypoxic conditions as well as in the microdissected samples. Cell proliferation and invasion were found to be decreased in one of the PC *INSIG2*-knockdown cell lines. The mRNA expression levels were also evidently higher in late-stage cancer compared to the early stage [64]. The hypoxia-inducible proapoptotic gene, *BNIP3,* was discovered to be downregulated in PDAC tissues as well as in cell lines. It showed resistance to both drugs gemcitabine and 5-fluorouracil, which led to a lower patient survival rate and a worse prognosis [62]. All these appealing results pointed out that these proteins play a vital role in pancreatic cancer progression and metastasis and they can act as a biomarker for diagnosis, prognosis, and therapy.

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