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Exosomal delivery of CRISPR/Cas9 complexes for targeted editing of KRAS^G12D in pancreatic cancer: prerequisites for clinical surgical application

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Abstract

Objective. To evaluate exosomes obtained from patients with ductal adenocarcinoma of the pancreas as carriers of CRISPR/Cas9 complexes for targeted editing of KRAS^G12D in pancreatic cancer and to test their stability, editing efficiency, and applicability for endoscopic or intraoperative administration.

Materials and methods. Biological material obtained from 42 patients with pancreatic ductal adenocarcinoma included tumor tissue, ascites fluid, and peripheral blood. Primary cultures of pancreatic ductal adenocarcinoma and organoids were created from tumor tissue. Exosomes were isolated from ascites fluid and culture supernatants, purified, and loaded with Cas9/sgRNA complexes targeting the KRAS^G12D mutation. Vesicle characterization included nanoparticle tracking analysis, transmission electron microscopy, and Western blotting. Editing efficiency and functional changes were studied in pancreatic ductal adenocarcinoma cells obtained from patients. To assess surgical feasibility, an intra–tumor endoscopic ultrasound fine–needle injection model was used with the introduction of DiI–labeled exosomes into a collagen–Matrigel hydrogel block (3:1).

Results. Exosomes were obtained with a high yield of $(1.6 \pm 0.3) \times 10^{10}$ particles/ml with a diameter of (102 ± 9) nm, more than 90% of which were effectively labeled. The loading efficiency of Cas9/sgRNA complexes was (47 ± 5)%. In pancreatic ductal adenocarcinoma cultures (n = 12), delivery via exosomes resulted in (31.4 ± 4.2)% indels at the KRAS^G12D locus and a (48 ± 6)% reduction in KRAS protein expression. The viability of functionally edited cells decreased by 35%, apoptosis increased almost threefold, and migration ability decreased by 45%. Combined use with gemcitabine further reduced the viability of functionally edited cells by 25%. In the model of intratumoral endoscopic ultrasound–guided fine–needle injection, exosomes remained stable – (102 ± 9) nm before injection and (104 ± 11) nm after injection ($p > 0.05$) and within 24 hours were diffusely distributed in the tissue by 3–4 mm, confirming their suitability for surgical use.

Conclusions. Exosomes obtained from patients, a biocompatible platform for delivering CRISPR/Cas9 complexes to pancreatic ductal adenocarcinoma tumor cells, provide effective editing of KRAS^G12D and suppression of tumor activity. Validation of the intratumoral endoscopic ultrasound–guided fine–needle injection model demonstrates the feasibility of translating this strategy into clinical intratumoral treatment.

Keywords: pancreatic ductal adenocarcinoma; KRAS^G12D; CRISPR/Cas9 complexes; exosomes; gene editing; intratumoral endoscopic ultrasound injection model; intratumoral therapy; translational oncology.

Pancreatic ductal adenocarcinoma (PDAC) remains one of the malignant tumors with the highest mortality rate, and the five–year survival rate for this pathology does not exceed 10%, despite advances in surgical techniques and systemic therapy [1, 2]. The main reasons for this unfavorable prognosis are late diagnosis, a pronounced desmoplastic stromal component that prevents the penetration of drugs, and the almost universal presence of oncogenic mutations in the KRAS gene [3, 4], among which the KRAS^G12D mutation is found in more than 90% of patients with PDAC and plays a key role in the initiation of the tumor process, its progression, and the development of resistance to chemotherapy [5, 6]. Therefore, strategies aimed at targeted editing of KRAS^G12D are of primary clinical importance.

CRISPR/Cas9 genome editing technology is currently considered a powerful tool for targeted inactivation of oncogenic drivers, in particular KRAS^G12D [7]. Preclinical studies have shown that editing KRAS with CRISPR/Cas9 complexes in vitro reduces proliferative activity, induces apoptosis, and increases the sensitivity of PDAC cells to chemo-

therapeutic agents [8, 9]. However, the main barrier to clinical implementation remains the lack of a safe and effective delivery system capable of penetrating dense stromal tissue and reaching tumor cells [10].

Adeno–associated viruses are being actively researched for CRISPR/Cas9 delivery, but they have low packaging capacity, potential immunogenicity, and nonspecific tropism, which limits their use for this purpose [11]. Lipid nanoparticles, which are effective for hepatic delivery, demonstrate low tropism, rapid clearance, and limitations due to the dense stromal barrier in the pancreas [12]. Thus, there is a critical need for alternative carriers that combine biocompatibility, the ability to transport ribonucleoprotein complexes, and the possibility of integration into surgical or endoscopic techniques.

Exosomes—small extracellular vesicles naturally secreted by cells—have recently attracted attention as promising carriers for the delivery of nucleic acids and proteins [13, 14]. They are intrinsically biocompatible, have low immunogenicity, and are capable of overcoming biological barriers, making them attractive for cancer therapy. Exosomes obtained from patients can be modified to transport CRISPR/Cas9 complexes, which is important because it enables personalized and tumor–specific approaches [15]. In addition, exosome delivery can be integrated into existing clinical protocols, in particular by endoscopic ultrasound–guided fine–needle injection or intraoperative administration during pancreatic resection, thereby creating a direct bridge between the laboratory and the operating room [16, 17].

In this context, we hypothesize that exosomes obtained from PDAC patients can serve as natural carriers for CRISPR/Cas9 complexes targeting KRAS^G12D. Combining experimental in vitro editing in cells derived from patients with a model of endoscopically guided ultrasound–guided fine–needle injection into a gel mimicking the stromal environment aimed to demonstrate both the efficiency of CRISPR/Cas9 complex delivery using exosomes and its translational potential for surgical oncology.

Research objective: to evaluate exosomes obtained from PDAC patients as carriers of CRISPR/Cas9 complexes for targeted editing of KRAS^G12D in pancreatic cancer and to test their stability, editing efficiency, and applicability for endoscopic or intraoperative administration.

Materials and methods

Biological samples were obtained from 42 patients with histologically confirmed PDAC who underwent surgical removal or diagnostic procedures between January 2023 and December 2024 at clinical centers affiliated with the Ukrainian Biobank Association in Austria. All patients provided written informed consent to participate in the study, which was approved by the institutional ethics committee of the Ukrainian Association of Biobanks (protocol No. 2003/2021 dated January 11, 2025) and conducted in accordance with the principles of the Helsinki Declaration and the European Union's General Data Protection Regulation.

Depending on clinical availability, three types of biological material were collected.

Fresh tumor samples (n = 42) were obtained during surgical resections of the pancreas. Immunohistochemical verification (CK7, CK19, Ki–67, p53) and genetic sequencing of KRAS mutations were performed in the research laboratory of the Austrian branch of the Ukrainian Biobank Association in Graz. Objective: to establish primary PDAC cell cultures derived from patients and organoids containing patient–specific mutations, including KRAS^G12D.

Rationale: Tumor tissue provides the most reliable representation of the PDAC molecular landscape, allowing direct testing of CRISPR/Cas9–mediated editing in cells that accurately reflect the patient's mutational background.

Ascites fluid (n = 28) was collected intraoperatively or by therapeutic paracentesis. Objective: To isolate circulating tu-

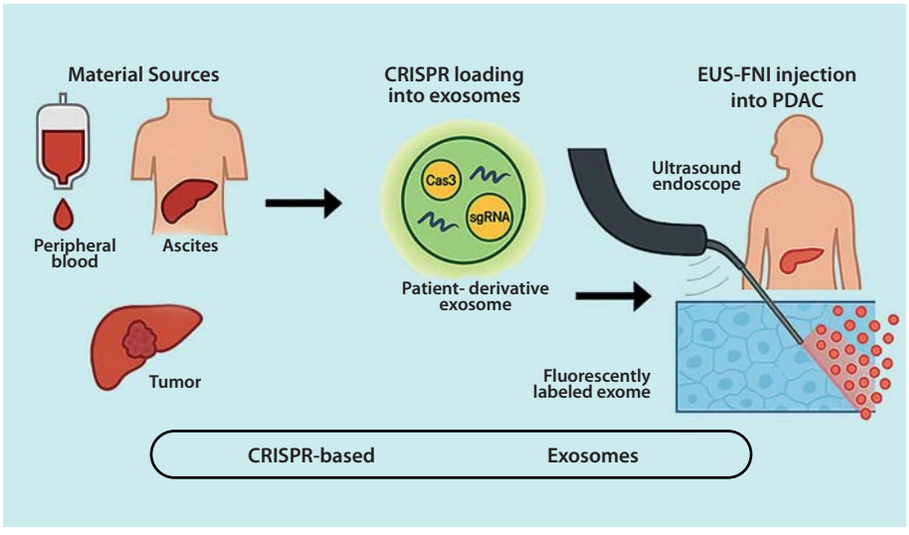

Рис. 1.
Схема трансляційного шляху від матеріалу, отриманого від пацієнтів, до інженерії екзосом і потенційного клінічного застосування при ПАПЗ.

mor cells and extracellular vesicles (exosomes). Rationale: Ascites fluid is a rich source of tumor–derived exosomes, which were used as natural carriers for Cas9/sgRNA complexes. Exosomes obtained from patients provided biologically relevant vesicles for evaluating CRISPR/Cas9 complex delivery via exosomes in conditions mimicking the patient's tumor microenvironment.

Peripheral blood in the amount of 10 ml per patient (n = 42) was collected in EDTA tubes before surgery or biopsy. Plasma and leukocyte fractions were processed at the Austrian branch of the Ukrainian Biobank Association. Objective: to isolate plasma for the detection of circulating tumor DNA and exosomes. Rationale: Blood–based material allows non–invasive confirmation of KRAS^G12D mutations outside the primary tumor and monitoring of exosomal load as potential biomarkers of therapeutic efficacy ("liquid biopsy"), which facilitates clinical translation.

A comprehensive translational platform was created by integrating tumor tissue, ascites fluid, and peripheral blood into a single workflow (*Fig. 1*). Diagnosis verification, immunohistochemical profiling, and genetic sequencing were performed exclusively for scientific purposes by the Austrian branch of the Ukrainian Biobank Association in Graz. This approach ensured a unique contribution of each type of material to the study: tumor tissue as an experimental platform for editing, ascites fluid as a personalized delivery source, and peripheral blood as a minimally invasive biomarker tool.

Biological material obtained from patients (tumor tissue, malignant ascites fluid, peripheral blood) was used as a source for isolating exosomes, which were then loaded with CRISPR/Cas9–sgRNA complexes targeting the KRAS^G12D mutation and labeled with DiI dye for visualization. Delivery was modeled using a fine–needle injection system under endoscopic ultrasound guidance into a hydrogel that mimicked PDAC stroma.

Cell isolation and cultivation

Primary tumor cells were isolated from fresh surgical tissue by mechanical dissociation and enzymatic digestion using type IV collagenase (, Gibco, Thermo Fisher Scientific, USA). The cells were cultured in DMEM/F12 medium supplemented with 10% bovine embryonic serum (Sigma–Aldrich, USA), antibiotics, and 1% GlutaMAX (Gibco). The cultures were maintained at 37 °C in a CO incubator with a carbon dioxide concentration of 5% (BINDER, Germany). The identity of the cells was confirmed by immunocytochemistry for cytokeratin 19 and vimentin (Abcam, UK).

Isolation and characterization of exosomes

Exosomes were isolated from malignant ascites fluid of PDAC patients and from tumor cell culture supernatants obtained from patients. After removing cell debris by sequential low–speed centrifugation, the vesicles were precipitated by ultracentrifugation at 100,000 rpm for 70 min (Beckman Coulter Optima XPN–100, USA) and further purified using ExoQuick reagent (System Biosciences, USA).

The characterization of exosomes included nanoparticle tracking analysis (NanoSight NS300, Malvern, UK), which confirmed a predominant size distribution of 80–120 nm, transmission electron microscopy (JEM–1400, JEOL, Japan), which revealed a typical cup–shaped morphology, and Western blotting for the exosome markers CD63, CD81, and TSG101 (antibodies from Abcam, UK).

These combined methods confirmed that the isolated vesicles were enriched with true exosomes with minimal contamination, providing a biologically relevant system for further experiments with CRISPR/Cas9 complex delivery.

Loading exosomes with Cas9/sgRNA complexes

CRISPR/Cas9 ribonucleoprotein complexes were assembled using recombinant SpCas9 nuclease (Integrated DNA Technologies, USA) and chemically synthesized sgRNA targeting KRAS^G12D (Synthego, USA). Exosomes were loaded with ribonucleoprotein complexes using electroporation (Neon Transfection System, Thermo Fisher Scientific, USA) according to optimized protocols. Loading efficiency was assessed using polymerase chain reaction (PCR) to detect sgRNA and Western blotting to detect Cas9 protein.

Fluorescent labeling of exosomes

To visualize exosomes, they were stained with the lipophilic fluorescent dye DiI (Vybrant DiI Cell–Labeling Solution, Invitrogen, USA), the excess of which was removed using Amicon Ultra–0.5 centrifugal filters with a 100 kDa threshold (Millipore, Germany). Fluorescently labeled exosomes were analyzed using a confocal microscope (Leica TCS SP8, Germany).

Verification of KRAS mutations

DNA from tumor tissue, exosomes, and plasma was extracted using the QIAamp DNA Mini Kit (QIAGEN, Germany). Mutations in exon 2 (codon 12) of the KRAS gene were analyzed by Sanger sequencing using an Applied Biosystems 3500 genetic analyzer (Thermo Fisher Scientific, USA), and the results were confirmed by droplet digital dPCR on a Bio–Rad QX200 system (Bio–Rad, USA). This dual approach ensured high sensitivity and specificity in detecting the KRAS^G12D mutation in both tissues and circulating material.

In vitro treatment with exosomes – Cas9/sgRNA

PDAC cells obtained from patients were cultured under standard conditions in DMEM/F12 medium supplemented with 10% bovine embryonic serum and antibiotics, and seeded into 6–well plates at a density of 1 10 cells per well. The cells were allowed to adhere overnight, after which the cultures were treated with exosomes labeled with DiI and loaded with Cas9/sgRNA ribonucleoprotein complexes specifically targeting the KRAS^G12D mutation. To ensure the reliability of the results, three experimental conditions were included: untreated cells (negative control for editing efficiency), cells treated with unloaded exosomes to assess the baseline effect of vesicular uptake, and cells transfected with Cas9/sgRNA ribonucleoproteins delivered using Lipo-

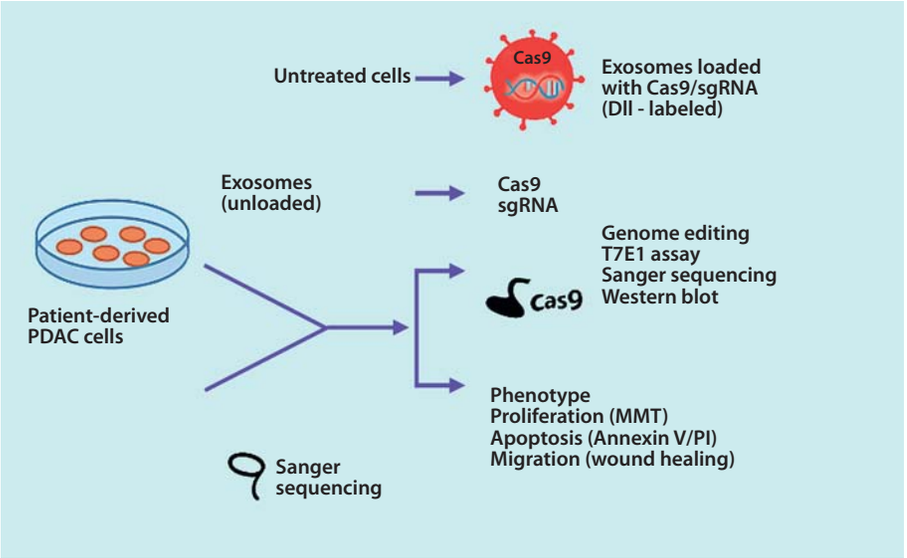

Fig. 2.
Schematic of the experimental workflow for the delivery of CRISPR/Cas9 complexes using exosomes into patient-derived pancreatic cancer cells.

fectamine CRISPRMAX (Thermo Fisher Scientific, USA) – positive control for editing efficiency.

After 72 hours of incubation, genomic DNA and protein were extracted from all experimental groups. The efficiency of genome editing was assessed using the T7 endonuclease I test to detect mismatch cleavage, which was confirmed by direct Sanger sequencing. Proteins were analyzed by Western blot using KRAS–specific antibodies (Abcam, UK) to determine the degree of reduction in mutant KRAS expression. These molecular analyses allowed direct comparison of the editing ability of exosome delivery with standard transfection.

In addition to verifying the editing, we investigated the functional effects of CRISPR/Cas9 treatment on cell behavior. Proliferation rate was assessed using the MTT assay, quantifying metabolic activity as an indicator of cell viability. Apoptosis induction was analyzed by annexin V and propidium iodide staining followed by flow cytometric analysis on a BD FACSCanto II system, thereby distinguishing between early and late apoptotic events. Cell migration was assessed using a wound healing assay, in which confluent monolayers were scratched with a sterile pipette tip, and wound closure was monitored for 48 hours using phase–contrast microscopy.

Taken together, this experimental setup (*Fig. 2*) allowed for a comprehensive assessment of the efficacy of molecular editing and the phenotypic consequences of delivering Cas9/sgRNA complexes via exosomes in PDAC cells derived from patients.

Combination with chemotherapy

To test the chemosensitivity of the cells, they were treated with gemcitabine (10 μM, Sigma–Aldrich, USA) alone or in combination with Cas9/sgRNA exosome treatment. Cell viability was measured after 72 hours using the XTT assay (Roche, Switzerland).

In vitro model of endoscopic ultrasound–guided fine–needle injection

To reproduce a clinically realistic local delivery route, an in vitro model of endoscopic ultrasound–guided fine–needle injection was created, which mimics the mechanical and diffusion properties of the desmoplastic stroma of the pancreas. To form the composite hydrogel, Matrixgel (Corning, USA) with reduced growth factor content was mixed with neutralized type I collagen from rat tail (Gibco, USA) in a 3:1 volume ratio on ice to prevent premature gel formation. The mixture was poured into flat molds (approximately 30 20 mm in size, final thickness 8–10 mm) and polymerized for 24 hours at 37 °C in a $_{CO}$ incubator. This composition and thickness were chosen to approximate the density and interstitial resistance of PDAC stroma, while allowing confocal optical sectioning along the entire injection trajectory.

Immediately prior to injection, the exosome preparations were labeled with DiI and quantified using nanoparticle tracking analysis for dose standardization (typically 1–2 10 particles/mL; injection volume 50–100 μL per site). The hydrogel block was placed in a phantom bath with water coupling to ensure acoustic coupling with a curved echoendoscope (Olympus GF–UCT180, Japan). Under real–time ultrasound imaging guidance, a 22 G EUS–FNA/FNI needle (EchoTip Ultra, Cook Medical, USA) was advanced through the working channel and inserted tangentially 4–6 mm into the gel, replicating the trajectory commonly used for intratumoral delivery to the pancreas. The injection was performed at a controlled rate (approximately 10–20 μl/s) to avoid retrograde reflux and promote parenchymal dispersion.

The spatial–temporal distribution of DiI–labeled exosomes was assessed after 2, 6, and 24 hours. For each time interval, the gel surrounding the injection site was mounted on a Leica TCS SP8 confocal microscope; three stacks (10–20 μm steps) were obtained perpendicular to the needle tra-

Клінічні та молекулярні характеристики досліджуваних пацієнтів із ПАПЗ (n = 42)

Параметр	Значення параметра	95% ДІ
Кількість пацієнтів, n	42	-
Вік, медіана (діапазон), роки	63 (48 – 77)	-
Стать, n (%)		-
ч	23 (54,8)	
ж	19 (45,2)	
Стадія згідно з системою стадіювання AJCC 8-го видання, n (%)		-
I	5 (11,9)	
II	21 (50)	
III	11 (26,2)	
IV	5 (11,9)	
Наявність пухлинної тканини, n (%)	42 (100)	-
Наявність асцитичної рідини, n (%)	28 (66,7)	-
Доступна периферична кров, n (%)	42 (100)	-
KRAS^G12D, n (%)		
пухлинна тканина	36 (85,7)	72,2 – 93,3
екзосоми, видалені з асцитичної рідини	24 (85,7)	68,5 – 94,3
плазмова кДНК	32 (76,1)	61,5 – 86,5
Інші варіанти KRAS, n		-
G12V	2	-
G12R	1	-
Імуногістохімічна верифікація, %		-
CK7/CK19	92	
аберантний p53	74	
Ki-67	30 – 70	
Встановлені культури первинної ПАПЗ, n (%)	34 (81)	-
Конкордантність KRAS^G12D, n (%)		;
пухлина і кДНК	30 (71,4)	к =0,39 p = 0,032
пухлина і екзосоми	22 (78,6)	к =0,42; p = 0,152

Примітка. ДІ – довірчий інтервал.

jectory, and maximum intensity projections were created to visualize radial spread. The penetration depth was defined as the furthest radial distance from the needle tip at which the signal intensity exceeded a predefined threshold (mean background value ± 3 SD), which yielded typical values of approximately 3–4 mm in this model. To ensure that the injection procedure did not compromise the integrity of the vesicles , aliquots were sampled immediately before loading and 30 min after injection; Size distribution and concentration were measured using nanoparticle tracking analysis (NanoSight NS300, Malvern, UK), and preparations showing a change in mean diameter of more than 10% were excluded from further analysis. Taken together, this workflow provided a reproducible laboratory platform that mimicked endoscopic intratumoral administration and allowed for quantitative measures of exosome stability and dispersion in a stroma–like matrix.

Statistical analysis

The data were analyzed using GraphPad Prism 10.0 (GraphPad Software, USA). The results of at least three independent experiments are presented as the mean ± standard deviation (SD). For statistical comparison, Student's t–test or one–way ANOVA (Analysis of Variance) with Tukey's post hoc test was used. Values of p less than 0.05 were considered statistically significant.

Results

The clinical and molecular characteristics of PDAC patients from whom biological material was collected were studied (*see table*).

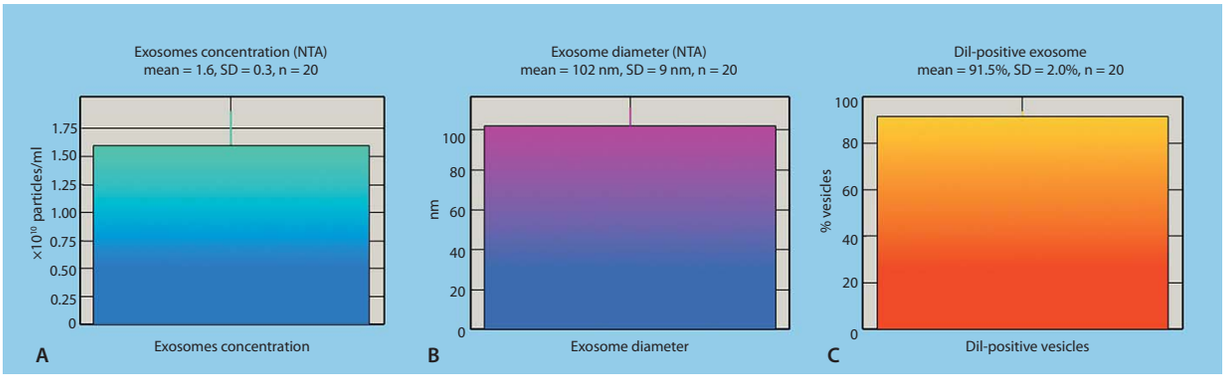

Fig. 3.
Isolation and characterization of exosomes: A - quantification of exosome yield by tracking assay B - distribution of exosomes by size determined by nanoparticle tracking analysis; C - the fraction of DiI-positive vesicles after fluorescent labeling.

KRAS sequencing confirmed that the KRAS^G12D mutation was dominant in tumor tissue, exosomes isolated from ascites fluid, and plasma cDNA. The detected concordance of the G12D mutation in solid, liquid, and vesicular compartments emphasizes its leading role in PDAC pathogenesis and justifies its selection as the primary target for CRISPR/Cas9 in this study.

Exosomes isolated from ascites fluid and supernatants of PDAC cell cultures had a mean concentration of (1.6 ± 0.3) × 10^{10} particles/ml (n=20) according to nanoparticle tracking analysis (*Fig. 3, A*). Their average hydrodynamic diameter (*Fig. 3, B)* was (102 ± 9) nm (n=20), which corresponded to true exosomes and was confirmed by transmission electron microscopy (characteristic round morphology up to cup–shaped). Western blotting confirmed strong expression of CD63, CD81, and TSG101 in 20 of the 20 preparations tested. After DiI labeling (*Fig. 3, B)*, the proportion of fluorescent vesicles suitable for tracking reached (91.5 ± 2.0)% (n=20), which ensured reliable tracking in subsequent in vitro experiments.

The average efficiency of loading Cas9/sgRNA complexes into exosomes by electroporation was (47 ± 5)% (n=20) with no aggregation detected by nanoparticle tracking analysis (*Fig. 4, A*). After exposure of PDAC cultures obtained from patients (n=12) to exosome–loaded complexes, the T7E1 assay demonstrated (31.4 ± 4.2)% indels at the KRAS^G12D locus (*Fig. 4, B)*, which was confirmed by Sanger sequencing; Western blot analysis showed that KRAS protein levels decreased by (48 ± 6)% (n=12) compared to untreated cells (*Fig. 4, B)*, confirming functional gene disruption. Control cells treated with unloaded exosomes showed (1.2 ± 0.8)% background indels (n = 6), while positive control transfection with CRISPRMAX lipofectamine showed (42.1 ± 5.3)% indels (n=12), confirming the reliability of the test.

Functionally, CRISPR/Cas9 complexes delivered by exosomes reduced cell viability by 35% ($p < 0.01$) compared to the control, as measured by the MTT assay. Flow cytometry analysis showed a significant increase in apoptosis: (29.3 ± 3.4)% of annexin V–positive cells, 11.2% in untreated cells, and 13.5% in cells treated with unloaded exosomes ($p < 0.001$). Wound healing analysis revealed a 45% reduction in migratory ability after 48 hours compared to the control ($p < 0.01$). Furthermore, when exosome editing was combined with gemcitabine, cell viability was further reduced by 25% compared to chemotherapy alone ($p < 0.05$), confirming the synergistic effect between KRAS inhibition and cytotox-

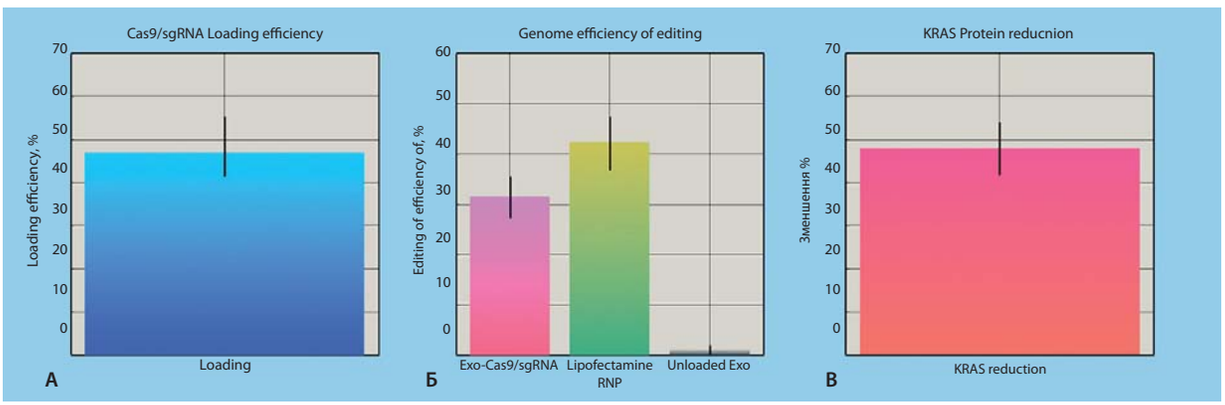

Fig. 4.
Efficiency of genome editing using exosomes: A - efficiency of loading Cas9/sgRNA complexes into exosomes by electroporation; B - efficiency of genome editing at the KRAS^G12D locus in patient-derived pancreatic cancer cells; C - reduction of KRAS protein expression in edited cells.

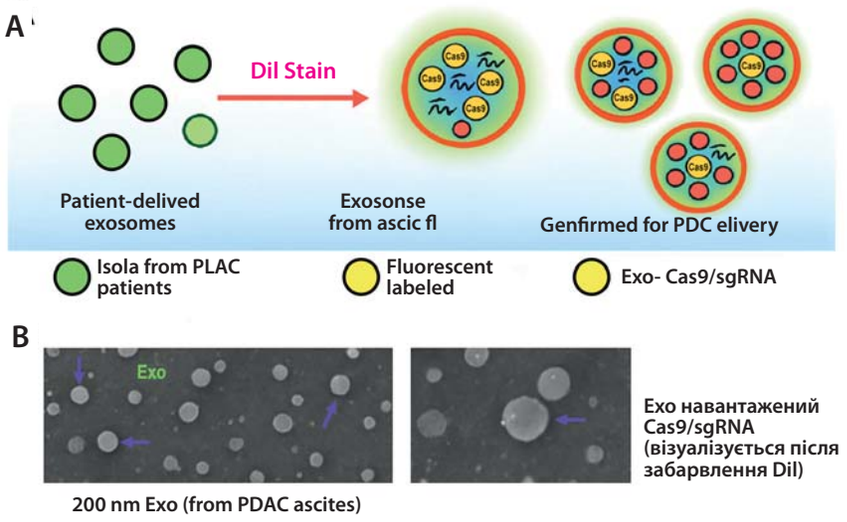

Fig. 5.
Characterization and engineering of patient-derived exosomes for CRISPR/Cas9 delivery: A - schematic representation of exosomes isolated from ascitic fluid of patients with PAPD with fluorescent labeled with DiI and loaded with with Cas9/sgRNA complexes targeting KRAS^G12D; B - images of exosomes obtained by transmission electron microscope (scale bar = 200 nm) on the left: native exosomes from ascitic fluid of patients with PAP, mean size (102 ± 9) nm, mean concentration of 1.6 × 10^{10} particles/ml, right: exosomes loaded with Cas9/sgRNA complexes visualized after DiI staining.

ic therapy. The workflow of exosome labeling, Cas9/sgRNA loading, and morphological validation by electron microscopy, shown in *Fig. 5,* confirms the integrity and functional suitability of patient–derived vesicles for use in genome editing.

To combine laboratory experiments with clinical application, an in vitro model of endoscopic ultrasound–guided fine–needle injection into collagen–Matrigel hydrogel was created to mimic the dense stromal architecture of PDAC. A block of type I collagen hydrogel – Matrixgel (ratio 3:1, thickness 8–10 mm), which simulated the PDAC stroma, was placed in a phantom chamber compatible with water–coupled ultrasound imaging. A curved echoendoscope (Olympus GF–UCT180, Japan) was used to insert a 22 G thin needle (EchoTip Ultra, Cook Medical, USA) into the gel under real–time ultrasound imaging, replicating the trajectory commonly used in clinical pancreatic biopsy or therapeutic injections.

DiI–labeled exosomes obtained from the ascites fluid of PDAC patients were standardized to a concentration of (1.8 ± 0.2) 10 particles/mL, and 50–100 µl of the suspension was injected at a rate of 10–20 µl/s to prevent reflux and ensure parenchymal dispersion. Confocal microscopy revealed a time–dependent radial distribution: after 2 hours, fluorescence was detected at a distance of up to (2.0 ± 0.3) mm from the needle track; after 6 hours, penetration spread to (3.6 ± 0.4) mm, and after 24 hours, stable fluorescence was maintained with a slight decrease in intensity (<15% signal decay compared to 6 hours). These values correspond to the reported interstitial resistance in PDAC stroma and indicate that exosomes are capable of penetrating the microenvironment, which is clinically significant.

Importantly, nanoparticle tracking analysis confirmed the integrity of exosomes during injections. The average diameter of the vesicles remained stable: baseline (102 ± 9) nm; after injection (104 ± 11) nm ($p > 0.05$), and particle recovery exceeded 92%, ruling out significant aggregation or degradation during needle passage. No differences in exosome stability were found in multiple replicates (n = 5 independent injections).

These results demonstrate that exosomes obtained from patients can be reliably delivered using standard endoscopic ultrasound–guided fine–needle injection without loss of integrity, retaining their ability to disperse in a stroma–mimicking environment at a depth of 3–4 mm. Considering that most PDAC lesions targeted by standard endoscopic ultrasound–guided fine–needle injection in the clinic have a diameter of 20–35 mm, the results obtained indicate that repeated or multi–site injections of exosomes can provide sufficient tumor coverage. Thus, this in vitro model provides clinically relevant confirmation of the concept of the feasibility of delivering CRISPR/Cas9 complexes based on exosomes as an adjunct to established endoscopic techniques for intratumoral therapy of pancreatic cancer (*Fig. 6)*.

Discussion

This study is translational, demonstrating the viability of the idea that exosomes obtained from patients can serve as effective and biologically compatible carriers for the delivery of CRISPR/Cas9 complexes targeting KRAS^G12D in PDAC, and differs from previous studies in a number of key aspects. First, the use of three clinically relevant sources—tumor tissue, malignant ascites, and peripheral blood—has enabled a comprehensive approach that combines experimental genome editing with surgical applicability. This is fundamentally different from most early work, which was based primarily on immortalized cell lines or xenografts and did not fully reflect the heterogeneity of patient material [18, 19].

Unlike viral vectors, particularly adeno–associated viruses, the exosomal approach overcomes limitations related to packaging capacity, the risk of integrative mutagenesis, and immunogenicity [20]. Lipid nanoparticles, which are effective for hepatic delivery, are characterized by low tropism to the pancreas and rapid clearance in the systemic circulation [21].

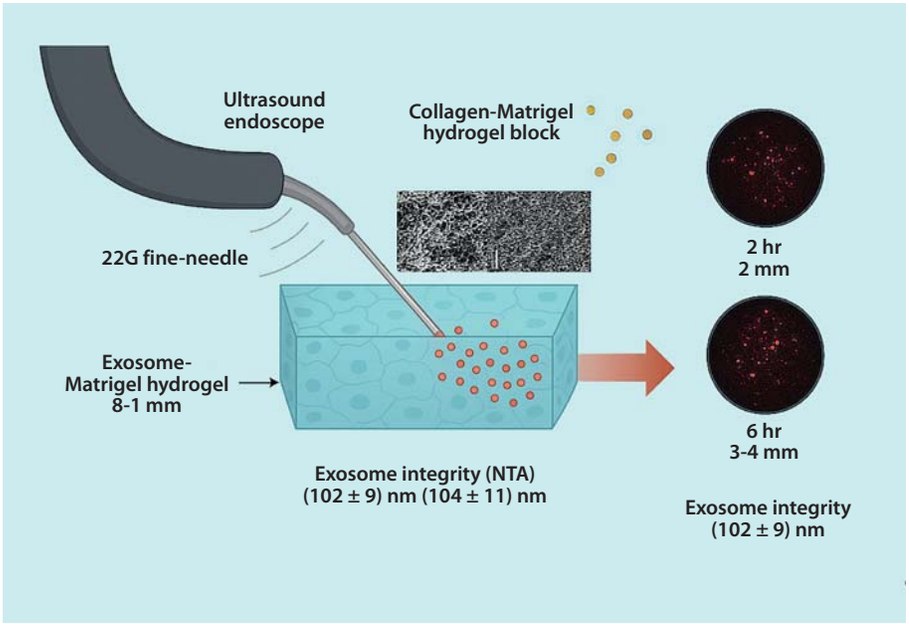

Fig. 6.
Schematic representation of the translational delivery model. Using an ultrasound endoscope (Olympus GF-UCT180), a 22 G fine needle (EchoTip Ultra, Cook Medical) is guided into the of the collagen-matrigel hydrogel, that mimics the stroma of the PAPC. Inset: light microscopy/macroscopic image of a collagen-matrigel hydrogel block used in the in the injection model.

In contrast, exosomes, especially those obtained from malignant ascites of PDAC patients, express surface proteins and adhesive molecules that promote selective uptake by tumor cells, providing natural tropism. Our results confirmed that exosomes can be reproducibly isolated in high yield – 1.6 10 particles/ml with a stable size – (102 ± 9) nm and high labeling efficiency of more than 90%, which ensured reliable delivery of Cas9/sgRNA complexes to primary PDAC cells.

Functionally, exosome–mediated CRISPR delivery resulted in an indel frequency of approximately 31% at the KRAS^G12D locus with a corresponding 48% reduction in KRAS protein levels. Although lipofectamine–mediated delivery of ribonucleoprotein complexes achieved slightly higher efficiency (approximately 42%), it is unsuitable for clinical application due to toxicity and the lack of safe in vivo protocols. The exosomal approach, on the other hand, provided biologically meaningful editing without compromising stability or biocompatibility. Functional consequences included a 35% reduction in cell viability, a nearly threefold increase in apoptosis, and a 45% reduction in migratory activity, and in combination with gemcitabine, an additional 25% reduction in viability. These results are consistent with previous reports of PDAC cell sensitization to chemotherapy upon KRAS inhibition [22], but demonstrate for the first time the possibility of using patient–derived exosomes as carriers.

The issue of clinical delivery is particularly important. An in vitro model of endoscopic ultrasound–guided fine–needle injection with collagen–Matrigel hydrogel, which mimics the density of the stroma, reproduces the mechanical and diffusion barriers of PDAC. Exosomes remained stable during needle passage – (102 ± 9) nm before injection and (104 ± 11) nm after injection ($p > 0.05$) – and demonstrated radial penetration of 3–4 mm within 24 hours. This directly confirms the possibility of integrating exosome–mediated genome editing into existing clinical procedures, such as endoscopic ultrasound fine–needle or intraoperative injections. Previous studies of the endoscopic ultrasound fine–needle injection model have focused primarily on viral or synthetic nanoparticles [23, 24]; to our knowledge, this study is the first to demonstrate the preservation of the structural integrity and functional capacity of exosomes after standard endoscopic injection.

Taken together, the results demonstrate a number of advantages: preservation of patient specificity and compatibility with the tumor microenvironment; surgical translatability thanks to the endoscopic ultrasound–guided fine–needle injection model; functional synergy with chemotherapy; and high biocompatibility.

Limitations include the in vitro study design and the absence of in vivo microenvironment factors (immune surveillance, vascular clearance, stromal remodeling). Future studies should include validation in orthotopic PDAC models, systemic safety assessment, and optimization of multifocal injections.

Conclusions

1. Exosomes obtained from PDAC patients can be effectively modified to carry CRISPR/Cas9 complexes targeting KRAS^G12D and provide significant genome editing and functional suppression of tumor cell growth.

2. Unlike viral or synthetic vectors, exosomes have demonstrated high stability, biocompatibility, and natural compatibility with the PDAC microenvironment. An in vitro model of endoscopic ultrasound–guided fine–needle injection confirmed that exosomal drugs maintain their integrity and are evenly distributed in the simulated stroma, highlighting their potential for minimally invasive surgical and endoscopic approaches, opening up the prospect of personalized in-

tratumoral therapy for pancreatic cancer.

Data and materials availability. Data sets generated and/or analyzed during this study are available from the corresponding author upon reasonable request.

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Conflict of interest. All authors declared no conflict of interest.

Consent to publish. All authors have read, approved the final version of the manuscript, and consented to its publication.

Ethical approval and consent to participate

The Bioethics Committee of the Ukrainian Biobank Association reviewed and approved the protocol for a pilot project on quality control of stem cells and cell lines, developed and implemented in partnership with the Ukrainian Biobank Association, the Institute of Cellular Biorehabilitation, the International Biobank, the Department of Surgery No. 1 of the Kharkiv National Medical University, and the Department of Medical Genetics of the Yerevan State Medical University (protocol No. 2003/2021 dated November 11, 2021).

References

1. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. CA Cancer J Clin. 2024 Jan-Feb;74(1):12-49. doi: 10.3322/caac.21820. Epub 2024 Jan 17. Erratum in: CA Cancer J Clin. 2024 Mar-Apr;74(2):203. doi: 10.3322/caac.21830. PMID: 38230766.
2. Mizrahi JD, Surana R, Valle JW, Shroff RT. Pancreatic cancer. Lancet. 2020 Jun 27;395(10242):2008-20. doi: 10.1016/S0140-6736(20)30974-0. PMID: 32593337.
3. Neesse A, Algül H, Tuveson DA, Gress TM. Stromal biology and therapy in pancreatic cancer: a changing paradigm. Gut. 2015 Sep;64(9):1476-84. doi: 10.1136/gutjnl-2015-309304. Epub 2015 May 20. PMID: 25994217.
4. Bailey P, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, et al. Genomic analyses identify molecular subtypes of pancreatic cancer. Nature. 2016 Mar 3;531(7592):47-52. doi: 10.1038/nature16965. Epub 2016 Feb 24. PMID: 26909576.
5. Waters AM, Der CJ. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. Cold Spring Harb Perspect Med. 2018 Sep 4;8(9):a031435. doi: 10.1101/cshperspect.a031435. PMID: 29229669; PMCID: PMC5995645.
6. Bannoura SF, Uddin MH, Nagasaka M, Fazili F, Al-Hallak MN, Philip PA, et al. Targeting KRAS in pancreatic cancer: new drugs on the horizon. Cancer Metastasis Rev. 2021 Sep;40(3):819-35. doi: 10.1007/s10555-021-09990-2. Epub 2021 Sep 9. PMID: 34499267; PMCID: PMC8556325.
7. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014 Nov 28;346(6213):1258096. doi: 10.1126/science.1258096. PMID: 25430774.
8. Lentsch E, Li L, Pfeffer S, Ekici AB, Taher L, Pilarsky C, et al. CRISPR/Cas9-Mediated Knock-Out of KrasG12D Mutated Pancreatic Cancer Cell Lines. Int J Mol Sci. 2019 Nov 14;20(22):5706. doi: 10.3390/ijms20225706. PMID: 31739488; PMCID: PMC6888344.
9. Lee W, Lee JH, Jun S, Lee JH, Bang D. Selective targeting of KRAS oncogenic alleles by CRISPR/Cas9 inhibits proliferation of cancer cells. Sci Rep. 2018 Aug 8;8(1):11879. doi: 10.1038/s41598-018-30205-2. PMID: 30089886; PMCID: PMC6082849.
10. Cheng Q, Wei T, Farbiak L, Johnson LT, Dilliard SA, Siegwart DJ. Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing. Nat Nanotechnol. 2020 Apr;15(4):313-20. doi: 10.1038/s41565-020-0669-6. Epub 2020 Apr 6. PMID: 32251383; PMCID: PMC7735425.
11. Hudry E, Vandenberghe LH. Therapeutic AAV Gene Transfer to the Nervous System: A Clinical Reality. Neuron. 2019 Mar 6;101(5):839-62. doi: 10.1016/j.neuron.2019.02.017. Erratum in: Neuron. 2019 Apr 3;102(1):263. doi: 10.1016/j.neuron.2019.03.020. PMID: 30844402; PMCID: PMC11804970.
12. Xu X, Wan T, Xin H, Li D, Pan H, Wu J, Ping Y. Delivery of CRISPR/Cas9 for therapeutic genome editing. J Gene Med. 2019 Jul;21(7):e3107. doi: 10.1002/jgm.3107. PMID: 31237055.
13. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. Science. 2020 Feb 7;367(6478):eaau6977. doi: 10.1126/science.aau6977. PMID: 32029601; PMCID: PMC7717626.
14. Kamerkar S, LeBleu VS, Sugimoto H, Yang S, Ruivo CF, Melo SA, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. Nature. 2017 Jun 22;546(7659):498-503. doi: 10.1038/nature22341. Epub 2017 Jun 7. PMID: 28607485; PMCID: PMC5538883.
15. EL Andaloussi S, Mäger I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov. 2013 May;12(5):347-57. doi: 10.1038/nrd3978. Epub 2013 Apr 15. PMID: 23584393.
16. Levy MJ, Chari ST, Wiersema MJ. Endoscopic ultrasound-guided celiac neurolysis. Gastrointest Endosc Clin N Am. 2012 Apr;22(2):231-47, viii. doi: 10.1016/j.giec.2012.04.003. Epub 2012 Apr 25. PMID: 22632946.
17. Chang KJ, Lee JG, Holcombe RF, Kuo J, Muthusamy R, Wu ML. Endoscopic ultrasound delivery of an antitumor agent to treat a case of pancreatic cancer. Nat Clin Pract Gastroenterol Hepatol. 2008 Feb;5(2):107-11. doi: 10.1038/ncpgasthep1033. PMID: 18253139.
18. McAndrews KM, Xiao F, Chronopoulos A, LeBleu VS, Kugeratski FG, Kalluri R. Exosome-mediated delivery of CRISPR/Cas9 for targeting of oncogenic KrasG12D in pancreatic cancer. Life Sci Alliance. 2021 Jul 19;4(9):e202000875. doi: 10.26508/lsa.202000875. PMID: 34282051; PMCID: PMC8321670.
19. Wan T, Zhong J, Pan Q, Zhou T, Ping Y, Liu X. Exosome-mediated delivery of Cas9 ribonucleoprotein complexes for tissue-specific gene therapy of liver diseases. Sci Adv. 2022 Sep 16;8(37):eabp9435. doi: 10.1126/sciadv.abp9435. Epub 2022 Sep 14. PMID: 36103526; PMCID: PMC9473578.
20. Balaraman AK, Babu MA, Moglad E, Mandaliya V, Rekha MM, Gupta S, et al. Exosome-mediated delivery of CRISPR-Cas9: A revolutionary

approach to cancer gene editing. Pathol Res Pract. 2025 Feb;266:155785. doi: 10.1016/j.prp.2024.155785. Epub 2024 Dec 19. PMID: 39708520.
21. Muangsanit P, Chailangkarn T, Tanwattana N, Wongwanakul R, Lekcharoensuk P, Kaewborisuth C. Hydrogel-based 3D human iPSC-derived neuronal culture for the study of rabies virus infection. Front Cell Infect Microbiol. 2023 Aug 25;13:1215205. doi: 10.3389/fcimb.2023.1215205. PMID: 37692167; PMCID: PMC10485840.
22. Nerger BA, Nelson CM. Bioprinting Cell-Laden Hydrogels for Studies of Epithelial Tissue Morphogenesis. Methods Mol Biol. 2024;2805:113-24. doi: 10.1007/978-1-0716-3854-5_7. PMID: 39008177.
23. Ren Z, Ahn EH, Do M, Mair DB, Monemianesfahani A, Lee PHU, et al. Simulated microgravity attenuates myogenesis and contractile function of 3D engineered skeletal muscle tissues. NPJ Microgravity. 2024 Feb 16;10(1):18. doi: 10.1038/s41526-024-00353-z. PMID: 38365862; PMCID: PMC10873406.
24. Sorzabal-Bellido I, Morales X, Cortés-Domínguez I, Esparza M, Grande L, Castillo P, et al. Tumor Organoids Grown in Mixed-Composition Hydrogels Recapitulate the Plasticity of Pancreatic Cancers. Gels. 2025 Jul 21;11(7):562. doi:

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