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BRIEF COMMUNICATION – Nature Medicine

Neoadjuvant CAN-2409 plus Prodrug in Combination with Standard of Care Chemoradiation for Borderline Resectable Pancreatic Adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains a deadly form of cancer because of its cold and immunosuppressive tumor microenvironment. A previous phase 1b clinical trial showed that treatment with CAN-2409 plus valacyclovir induced a significant increase in tumor infiltrating lymphocytes in the tumor. We report the findings of a randomized controlled phase 2a clinical trial of CAN-2409 plus valacyclovir with standard of care (SoC) for borderline resectable PDAC. Estimated median overall survival was 28.8 months in the CAN-2409 group versus 12.5 months in controls. There was systemic immune activation and formation of lymphocyte aggregates that resemble tertiary lymphoid structures in the test arm, but not in controls.

Although surgical resection of pancreatic ductal adenocarcinoma (PDAC) remains the only potential curative therapy, only 10-20% are resectable.[1] Patients who are unsuitable for surgery due to the location of their tumor nearby blood vessels may see positive results from surgery after undergoing chemotherapy and chemoradiation treatment beforehand. This is known as borderline resectable (BR) PDAC. Resection rates after standard of care (SoC) chemotherapy are 53-55%.[2] In BR PDAC, overall survival (OS), the gold standard for evaluating effectiveness of treatment in PDAC patients[3], is only 16.9 to 22.0 months from the beginning of systemic treatment[2], which is explained, in part, by the immunosuppressive tumor microenvironment (TME).

CAN-2409 (aglatimagene besadenovec) is a replication-defective adenovirus encoding the HSV-thymidine kinase gene. Local enzyme activity will convert orally administered valacyclovir (prodrug) into nucleotide analogs that result in immunogenic cell death of cancer cells, leading to in situ vaccination against the tumor, which is dependent on CD8+ T cells.[4-7] A phase 1b clinical trial of CAN-2409 in PDAC has shown that these findings can be replicated in patients[8], consistent with similar findings in prostate cancer and non-small cell lung cancer (NSCLC). Thus, CAN-2409 plus prodrug may convert the TME from an immunosuppressive 'cold tumor' into a 'hot tumor' state.[9,10]

The randomized, controlled phase 2a clinical trial of CAN-2409 plus prodrug in combination with SoC compared with SoC alone presented here was conducted in BR PDAC (ClinicalTrials.gov registration: NCT02446093). Following SoC induction chemotherapy, BR status was established upon review of the computer tomography (CT) scan (See **Supplementary Information).** Next, patients were enrolled and randomized to receive either CAN-2409 (2-3 intratumoral injections of $5x10^{11}$ vp) and valacyclovir (2g TID for 14 days, beginning 1-3 days after each CAN-2409 injection) plus SoC chemoradiation (n=7 test arm) or SoC chemoradiation alone (n=6 control arm). The first CAN-2409 injection was administered via endoscopic ultrasound 5-7 days prior to initiation of SoC chemoradiation, the second during chemoradiation, and the third was offered at tumor resection (**Supplementary Figure 1**). We used

descriptive statistics to explore the effects of CAN-2409 plus valacyclovir in combination with SoC compared to SoC alone.

Treatment with CAN-2409 plus prodrug was generally well tolerated; no dose limiting toxicities were reported, consistent with previous reports **(Supplementary Table 1).**[8,10]

Baseline characteristics were well balanced between the two arms (**Supplementary Table 2)**. Six out of seven patients in the test arm had tumors in the head or uncinate process versus three out of six patients in the control arm; once the tumor is classified as BR, anatomical tumor location does not influence prognosis.[11] In contrast, resection status does represent a prognostic factor. In our study 50.0% (3 out 6) had successful resection in the control arm, while 42.9% (3 out of 7) achieved resection in the test arm, thus providing a small prognostic advantage in the control arm (**Supplementary Table 3**). Of the unresected patients, four (2 in the test and 2 in the control arm) were classified as unresectable at re-staging prior to surgery and two were unresectable at time of surgery (both in the test arm). One patient in the control arm refused surgery for personal reasons.

Patient 2042 received two doses of CAN-2409, but was not resected due to liver metastases discovered during the attempted Whipple procedure; she only received salvage chemotherapy. The metastases apparently disappeared over time and successful primary tumor resection was performed 28 months from study initiation, without residual disease on operative exploration (**Supplementary Figure 2)**. She remained disease free for over one year, when recurrence prompted re-initiation of chemotherapy. She was still alive 54.2 months from study enrollment.

Survival analysis showed that, despite the lower proportion of patients able to undergo tumor resection in the test arm, median OS (mOS) in patients treated with CAN-2409+prodrug combined with SoC was markedly longer than in controls. At the time of data cutoff (March 29th, 2024), mOS was 28.8 months after enrollment in the test arm compared to only 12.5 months in SoC control arm (**Figure 1)**. mOS was 34.2 months after initiation of systemic chemotherapy in the test arm versus 15.9 months in the control arm; mOS in controls is consistent with published literature[2]. At 24 months, 71.4% of the patients in the

test arm were alive versus 16.7% in controls. Evaluation of CA19-9 levels, a biomarker reflecting tumor burden in PDAC, showed that progression occurred earlier in patients in the control arm, progression was not reversible by salvage chemotherapy, and patients died early after progression **(Figure 2)**. In contrast, time to progression was delayed in the test arm, CA19-9 levels were markedly reduced after salvage chemotherapy, and the patients stayed alive for more than 15 months after progression (**Figure 2).** The response to salvage chemotherapy in the test arm, but not in the control arm, is illustrated by doubling of survival after progression in the test arm (21.2 months in test arm versus 7.2 months in control arm, HR 0.34).

The clinical results may be explained by marked biological changes after CAN-2409+prodrug treatment. Paired tissue analysis demonstrated formation of lymphocyte aggregates (**Supplemental Figure 3)** comprised of CD8+ T cells, CD20+ B cells, and CD11c+ dendritic cells in the test arm, but not in controls **(Supplementary Figure 4)**. Immunofluorescence showed T cell proliferation and cytotoxicity **(Supplementary Figure 4)**. These changes were associated with tumor necrosis. In two pancreatic tissue samples obtained 28.4 months and 3.3 months after initial treatment, respectively, we observed organization of lymphocyte aggregates resembling tertiary lymphoid structures (TLS). The presence of TLS is associated with improved prognosis across solid tumors. There was also evidence of systemic immune activation in the test arm, but not in the control arm. Proteomic analysis of serial peripheral blood samples showed an increase in serum levels of granzyme B, granzyme H, IFNγ, and IL-10 (data not shown).

The results of the randomized, controlled phase 2a clinical trial of CAN-2409+prodrug (valacyclovir) in BR PDAC presented here show 1) mOS from enrollment of 28.8 months in the test arm compared to 12.5 months in the control arm, with a survival benefit in the test arm even in patients who were not able to undergo tumor resection. mOS was 34.2 months from initiation of systemic chemotherapy in the test arm versus 15.9 months in the control arm; 2) A decrease in serum levels of CA19-9 after salvage

chemotherapy in patients with progressive disease in the test arm, but no decrease in CA19-9 levels and early death in patients with progressive disease in the control arm despite salvage chemotherapy; and 3) Formation of lymphocyte aggregates resembling TLS in the TME associated with systemic immune activation in the test arm, but not in the control SoC arm.

A limitation of this clinical trial is its size. It appears unlikely, however, that the marked increase in mOS observed in the test arm can be explained by chance, placebo effects, or regression to the mean. The observed increase in mOS in the test arm is supported by landmark analysis, which showed that 71.4% of the patients in the test arm were still alive 24.0 months after enrollment versus only 16.7% in the control arm. Moreover, there was an unusual clinical observation in a patient with metastatic PDAC in the test arm who was still alive and in follow-up 54.2 months after study enrollment. It is also unlikely that the differences in mOS between the treatment arms can be explained by an abnormally short mOS in the control SoC group; the observed mOS of 15.9 months after initiation of systemic chemotherapy in the control arm is consistent with a recent meta-analysis[2]. Finally, it appears unlikely that the differences in mOS between the treatment arms can be explained by differences in prognostic factors at baseline. More patients in the test arm had a tumor in the head or uncinate process of the pancreas compared to the control arm; after establishing a diagnosis of BR PDAC, anatomical tumor location does not influence prognosis[11]. The only relevant imbalance between the two groups was the difference in resection rate, representing a small prognostic disadvantage for the test arm.

The increase in mOS observed in the test arm may be explained by the immunological changes induced by CAN-2409+prodrug treatment. This notion is supported by systemic immune activation and histologic changes observed in the pancreatic TME after CAN-2409+prodrug treatment, but not after SoC alone. These results indicate that viral immunotherapy with CAN-2409+prodrug may convert the TME from an immunosuppressive 'cold tumor' into a 'hot tumor' state, extending previous findings in phase 1b clinical trials of CAN-2409 in PDAC[8], prostate cancer[9] and NSCLC[10]. In a very recent phase 2a clinical trial in

patients with therapy-resistant, mostly metastatic NSCLC, we observed a marked increase in mOS after CAN-2409+prodrug treatment compared to historical controls treated with docetaxel chemotherapy[12], consistent with the results presented here in PDAC. This data provides the rationale for a larger, randomized controlled clinical trial of CAN-2409 in BR PDAC.

Legends to the Figures

Figure 1. Kaplan-Meier analysis of survival since enrollment by study arm. Censored = alive, follow-up ongoing.

Figure 2. Serum levels of CA19-9 in relationship to survival by treatment arm. Green lines represent patients who were still alive at data cutoff, red lines represent data from patients who had died. An increase in CA19-9 levels indicates progressive disease (PD), followed by SoC salvage chemotherapy in both arms. PD occurred later, in less patients, and there was a biological and/or clinical response to salvage chemotherapy in the test arm.

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Online methods

Study Design

NCT02446093 is an open label, multicenter, randomized, phase 2a clinical trial in patients with borderline resectable pancreatic adenocarcinoma (BR PDAC) to evaluate safety and efficacy of CAN-2409+prodrug added to SoC chemoradiation (CR) and surgery (test arm) compared to SoC CR and surgery (control arm). Patients were randomized 1:1 into either test or control arms. Patients were enrolled after receiving at least 2 months of SoC induction chemotherapy (investigator's choice of either FOLIFIRINOX or gemcitabine/nab-paclitaxel). BR status was established centrally by principal surgical investigator upon review of the CT scan at enrollment. The control arm received SoC chemoradiation followed by surgery.

Test arm patients received 2 to 3 injections of CAN-2409 at a dose of $5x10^{11}$ viral particles followed by a 14-day course of valacyclovir (2 grams orally three times a day) that was initiated 1-3 days following CAN-2409 injection (see supplementary figure 1). Intravenous acyclovir at a dose of 10mg/kg was substituted for valacyclovir if the patient was not able to tolerate oral medications; one patient (patient 2062) received acyclovir in this trial. The first injection of CAN-2409 was delivered intratumorally by endoscopic ultrasound (EUS) or CT guidance 5-7 days prior to the initiation of SoC chemoradiation, the second injection was administered during the chemoradiation regimen, and the third at the conclusion of surgical resection into the tumor bed or the tumor if not resectable, as previously described.[9]

Institutional review boards in all participating institutions approved the protocol and informed consent documents. Specific written informed consent was obtained from each patient prior to enrollment.

Patient Population

Eligible patients included males and females ≥18 years of age with an ECOG performance of 0-2 and a diagnosis of PDAC adequately treated with induction chemotherapy for at least 2 months such that they are candidates for CR followed by surgery. Patients were deemed to be in adequate health to undergo major surgery (pancreaticoduodenectomy). Tumor must have been accessible for injection and was classified as BR after central review by surgical investigators, based on pre-induction chemotherapy imaging. Resection may have included major vascular resection with reconstruction as needed.

BR classification criteria included:

- No distant metastasis or lymph node involvement outside the planned resection field
- Venous involvement of the superior mesenteric vein (SMV) or portal vein (PV) with distortion or narrowing of the vein or occlusion of the vein with suitable vessel proximal and distal, allowing for safe resection and replacement
- Gastroduodenal artery encasement up to the hepatic artery with either short segment encasement or direct tumor abutment of the hepatic artery, without extension to the celiac axis
- Tumor abutment of the superior mesenteric artery (SMA) not to exceed >180 degrees of the circumference of the vessel wall

Laboratory inclusion criteria included AST ≤ 3x upper limit of normal, platelets > 100,000/mm3, WBC > 3000/mm3, ANC > 1500/mm3, serum creatinine <2 mg/dl, and calculated creatinine clearance >10 ml/min.

Assessments

Clinical assessments with physical exam and toxicity assessment were performed according to the following schedule: week 1 before start chemoradiation (required for test arm only). For control and test arms: week 2-3 (if test arm, prior to second injection, week 3-4 (if test arm, approx. 2 weeks post second injection); during re-staging after chemoradiation completed prior to surgical resection; and 2-3 weeks after surgery. Standard of care follow-up with collection of pancreatic cancer status and new medical problems continued every 3-4 months after surgery for 2 years then approximately every 6 months for 3 years.

Toxicity was assessed using the National Cancer Institute common terminology criteria for adverse events (CTCAE) version 4.0. Dose-limiting toxicities (DLTs) were defined as any grade 4 toxicity or a grade 3 toxicity requiring interruption in SoC therapy for more than 2 weeks.

CA 19-9 response was presented as U/mL for all available timepoints at or after enrollment. All post-treatment timepoints are presented as months relative to enrollment date. Patients without CA 19-9 measurements at baseline or post-treatment were excluded from the analysis.

Hematoxylin and Eosin (H&E) staining

Tumor tissue was obtained either from diagnostic biopsies obtained at the time of diagnosis or at any time prior to study initiation or from tumor resection obtained at the time of surgery. Tissue was processed by the local histopathology laboratory, embedded in paraffin blocks and sectioned at 4µm, mounted on positively charged slides and dried at 58°C for 60min.

Hematoxylin and Eosin (H&E) staining was performed by iHisto Inc. (Salem, MA). Briefly, following deparaffination and hydration, staining was conducted using an autostainer system (Leica, ST5020-CV5030). After 3 minutes of room temperature incubation in Hematoxylin, slides were rinsed, and

differentiation was performed in a bluing reagent for 2 minutes and washed. After brief immersion in alcohol, slides were incubated in Eosin Y for 1 minute and dehydrated in absolute alcohol, then cleared in xylene. Slides were mounted with mounting media (Leica, MICROMOUNT) and whole slide scanning (40x) was performed on Motic Easy scan Infinity.

Immunofluorescence

Immunofluorescence staining was performed by iHisto Inc. (ihisto.io), Boston. Briefly, following deparaffination and washing three sequential cycles of antigen retrieval and incubation with primary and secondary antibodies were performed. In each cycle, slides were retrieved with antigen retrieval solution at 110°C for 15 min. Slides were cooled and washed in Tris-buffered Saline and Tris-buffered Saline + Tween20, treated with a peroxidase blocking solution for 10 minutes at room temperature and, after rinsing, incubated overnight at 4°C with the first primary antibody listed in Table 1. All primary antibodies were diluted in 2.5% normal goal serum. For all slides except for Group 2, after washing, secondary antibodies were incubated at room temperature for 1 hour. After washing, slides were incubated at room temperature for 10 minutes in a tyramide signal amplification (TSA) working solution. All slides including Group 2 slides were washed and conjugated secondary antibodies were incubated at room temperature for 1 hour. This process was repeated 3 times. After final washing, slides were stained with Sudan Black for 20 minutes to quench background autofluorescence, then washed in running water for 15 minutes, then in TBS and TBST. Slides were counterstained with DAP and coverslipped using Fluoroshield. Whole slide scanning was performed at 52x:0.172/pixel and image analysis was performed in Halo software.

Methods Table 1: Primary and Secondary Antibody Information

Primary Antibody	Dilution	Secondary Antibody
Group 1		
CD8 (70306, Cell Signaling Technology)	1:1000	Goat anti-mouse IgG HRP
		TSA 488
Pan-CK (nbp2-29429; Novus Biologicals)	1:200	Goat anti-mouse IgG 750
Ki67 (ab16667; Abcam)	1:50	Goat anti-rabbit IgG 555
Group 2 (No TSA)		
GzmB (14-8889-80; Invitrogen)	1:100	Goat anti-rat IgG 555
CD8 (70306; Cell Signaling Technology)	1:200	Goat anti-mouse IgG 750
CD3 (NB600-1441; Novus Biologicals)	1:100	Goat anti-rabbit IgG 647
Group 3		
CD16 (AB203883; Abcam)	1:1000	Goat anti-rabbit IgG HRP
		TSA 555
CD3 (NB600-1441; Novus Biologicals)	1:100	Goat anti-rabbit IgG 647
CD56 (3576; Cell Signaling Technology)	1:200	Goat anti-mouse 750
Group 4		
CD4 (93518; Cell Signaling Technology)	1:1000	Goat anti-rabbit IgG HRP
		TSA 555
CD11c (45581; Cell Signaling Technology)	1:1000	Goat anti-rabbit IgG HRP
		TSA 488
CD20 (48750; Cell Signaling Technology)	1:200	Goat anti-rabbit 647

Statistical Analysis

Overall survival (OS) is defined as the time from date of enrollment to the date of death or censored at the time of last available follow-up record, and OS after progression is defined as the time from date of progression to the date of death or censored at the time of last available follow-up record. The Kaplan-Meier method was used to estimate the landmark survival rate and the median. The two-sided 95% CI for the median and survival rate were calculated using the complementary log-log transformation method if applicable.

Manuscript figures

Figure 1

a.

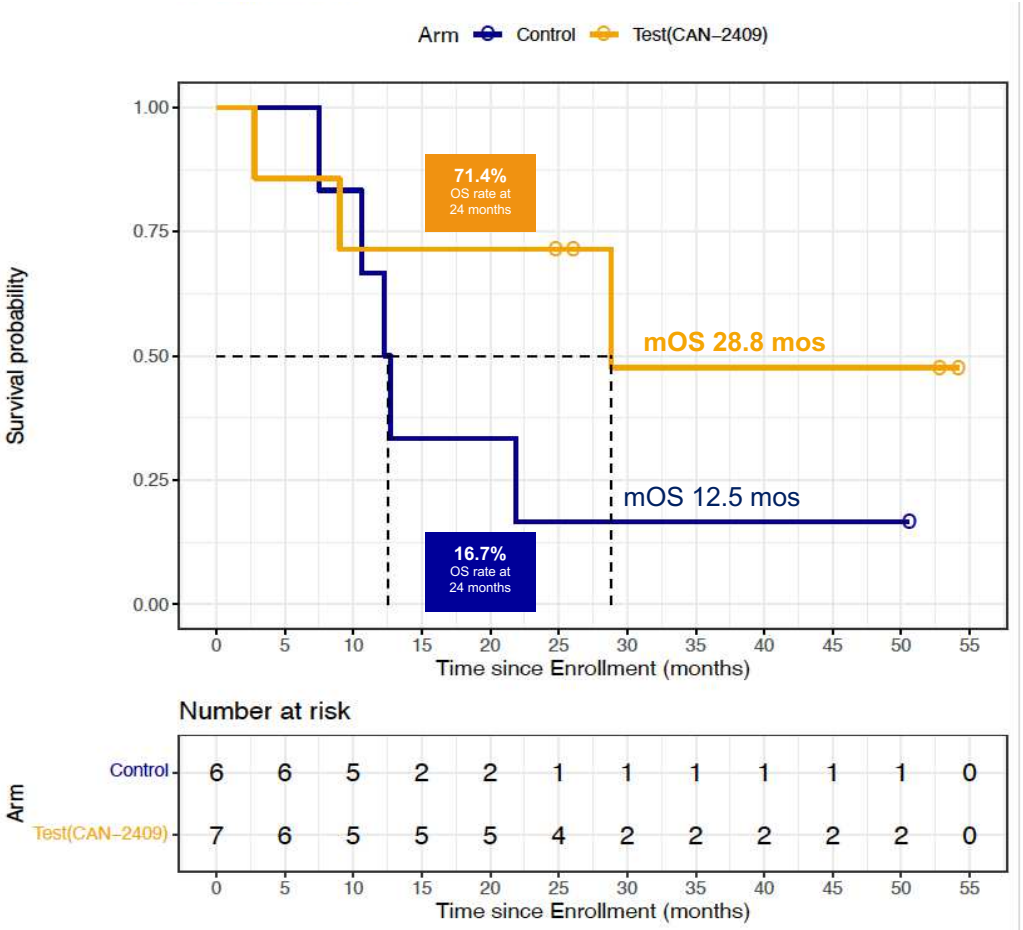
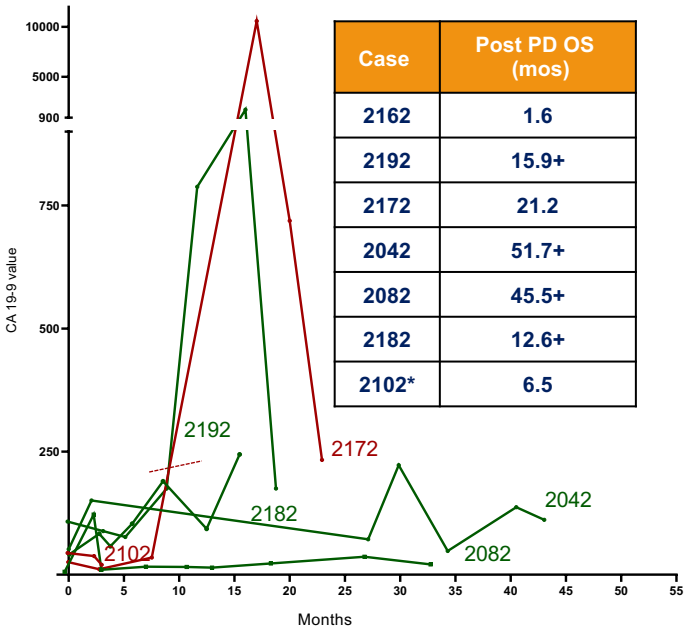

Arm
Control
Test(CAN–2409)
Survival probability
1.00
0.75
0.50
0.25
0.00
71.4%
OS rate at
24 months
mOS 28.8 mos
mOS 12.5 mos
16.7%
OS rate at
24 months
0
5
10
15
20
25
30
35
40
45
50
55
Time since Enrollment (months)
Number at risk
Arm
Control
6 6 5 2 2 1 1 1 1 1 1 0
Test(CAN–2409)
7 6 5 5 5 4 2 2 2 2 2 0
Time since Enrollment (months)

Figure 2

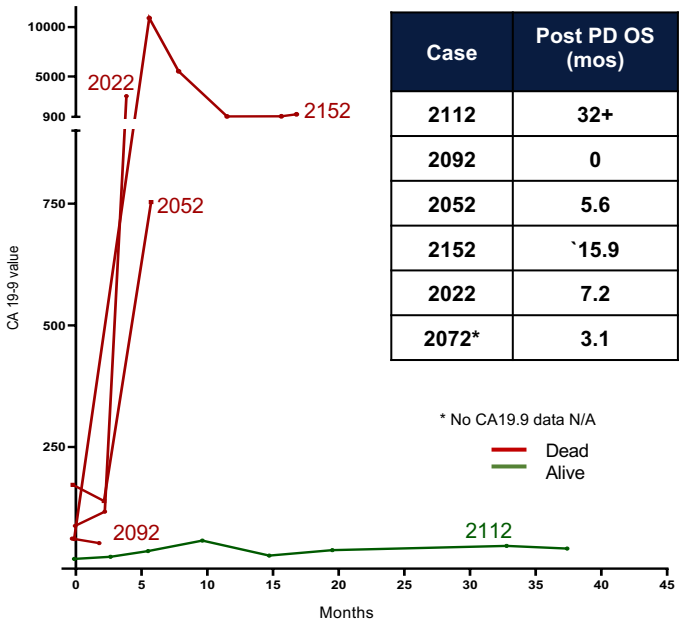
a.

CAN-2409 arm



Case	Post PD OS (mos)
2162	1.6
2192	15.9+
2172	21.2
2042	51.7+
2082	45.5+
2182	12.6+
2102*	6.5

Control arm



Case	Post PD OS (mos)
2112	32+
2092	0
2052	5.6
2152	`15.9
2022	7.2
2072*	3.1

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- PaTK02SupplementalFiguresTablesFinal.pdf