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Effects of Hypercapnia, an Element of Obstructive Respiratory Disorders on Pancreatic Cancer Chemoresistance and Progression

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Abstract

Background—Chronic obstructive respiratory disorders (ORD) are linked to increased rates of cancer related deaths. Little is known about the effects of hypercapnia (elevated CO_2) on pancreatic ductal adenocarcinoma (PDAC) development and drug-resistance.

Study Design—Two PDAC cell-lines were exposed to normocapnic (5% CO_2) and hypercapnic (continuous/intermittent 10% CO_2) conditions, physiologically similar to patients with active ORD. Cells were assessed for proliferation rate, colony formation, and chemo/radiotherapeutic efficacy. In a retrospective clinical study design, patients with PDAC who have undergone pancreatic resection between the years of 2002–2014 were reviewed. Active smokers were excluded in order to remove possible smoking-related pro-tumorigenic influences. Clinical data, pathological findings, and survival endpoints were recorded. Kaplan-Meier and Cox regression analyses were performed.

Results—Exposure to hypercapnia resulted in an increased colony formation and proliferation rate, *in-vitro* in both cell lines (MIA-PaCa-2:111% increase and Panc-1:114% increase, $P<0.05$). Hypercapnia exposure induced a 2.5-fold increase in oxaliplatin resistance ($P<0.05$) in both cell lines and increased resistance to ionizing radiation in MIA-PaCa-2 cells ($P<0.05$). Five hundred and seventy-eight patients were included [52% males, median age was 68.7 years (IQR 60.6–76.8 years)]. Cox regression analysis, assessing TNM-staging, age, gender and ORD status, identified ORD as an independent risk factor for both overall survival (HR 1.64, 95%CI 1.2–2.3, $P<0.05$) and disease-free survival (HR 1.68, 95%CI 1.06–2.67).

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Conclusions—PDAC cells exposed to hypercapnic environments, common to patients with ORD, showed tumor proliferation, radioresistance and chemoresistance. Patients with a history of ORD had a worse overall prognosis, suggesting that hypercapnic conditions play a role in the development and progression of PDAC and stressing the need for patient-tailored care.

Precis

Exposure of pancreatic cancer cells to 10% CO2 hypercapnic environment resulted in increased proliferation, radioresistance, and oxaliplatin chemoresistance. Clinically, pancreatic cancer patients with history of obstructive respiratory diseases had increased decreased survival and increased rates of recurrence.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) has become the 3rd leading cause of cancer mortality in the U.S., estimated to account for over 56,000 new cases in 2019 [1]. Smoking, a major risk factor for development of PDAC [2], also plays a key role in the development of pathologic respiratory conditions (*e.g.*, emphysema and chronic bronchitis [3]) resulting in hypoxia and hypercapnia. Both hypoxia and hypercapnia, combined with acidosis, have been described as common features of the tumor microenvironment [4–6]. Hypoxia, specifically, has been reported in numerous studies as a strong facilitator of PDAC resistance to therapy [7, 8]. However, studies of the biologic effects of hypercapnia are limited. In some reports, pathophysiologic high levels of CO_2 were associated with increased tumor proliferation and resistance to chemotherapy in other cancer types [5, 9]. Furthermore, some recent studies suggest hypoxia, hypercapnia, and respiratory acidosis as being important modulators and suppressors of anti-cancer immune responses [8, 10, 11].

Clinically, large studies have alluded to a possible link between obstructive respiratory diseases (ORD, such as bronchitis, asthma and emphysema) and increased rates of non-tobacco related cancers [12, 13]. Additionally, a few studies investigating obstructive sleep apnea, which is also characterized by transient hypercapnia and hypoxia have also suggested a connection to increased cancer risks [14, 15]. In regards to chronic exposure of lung parenchyma to smoke, significant resultant changes in respiratory physiology include prolonged hypercapnia, transient hypoxia, and chronic inflammation which can lead to permanent destruction of lung tissues [16, 17], and the development of a chronic obstructive pulmonary disease (COPD) [3]. These pathologic changes can systemically affect distal tissue oxygenation and carbon dioxide accumulation [18]. This notion is supported by several large population studies which support the association of hypoxic/hypercapnic respiratory conditions with cancer susceptibility [19, 20]. In fact, Kornum et al [12] have shown an association of COPD with increased cancer rates, including non-tobacco related cancers, in a large Danish nationwide cohort (N=236,494). Similar findings were also supported by a long-term prospective study, showing increased risks for breast cancer, prostate cancer, non-melanoma skin cancers and hematological malignancies [13]. Herein, we tested whether the effects of chronic and repeated exposure to high levels of CO_2, as seen in patients with ORD, were associated with worse clinical outcomes in patients with PDAC. We also hypothesized that such exposures would result in a more aggressive and therapeutic resistant cancer phenotype.

MATERIALS AND METHODS

Cell culture & CO_2 Exposure

PDAC cell lines (MIA-PaCa-2 and PANC-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and were grown in standard DMEM based media, supplemented with 10 % FBS, 1 % Penicillin-Streptomycin, 1 % L-Glutamine (Thermo Fisher Scientific, Waltham, MA, USA). All cells were regularly tested for mycoplasma using LookOut® Mycoplasma PCR Detection Kit (Millipore-Sigma, St. Louis, MO, USA). The cells were cultured in normal incubator conditions (*i.e.*, 37 °C in 5 % humidified CO_2 incubators), unless otherwise indicated. To mimic hypercapnia, cultures were subjected to either constitutive 10 % CO_2 environment or "intermittent" exposure (three consecutive hours daily), as indicated. A control arm of regular 5 % CO_2 with no exposure to hypercapnic conditions was also assessed.

Cell proliferation and survival assays

Cells were seeded in 96-well plates in triplicate and were separated into three groups after 24 hours (5 % CO_2 Control, Continuous 10 % CO_2, Intermittent 10 % CO_2 exposure). Cells were treated with either ionizing radiation or chemical cellular stressors such as DNA damaging chemotherapeutics (gemcitabine and oxaliplatin, Millipore-Sigma, St. Louis, MO, USA). Cell count was assessed using double stranded DNA quantification with PicoGreen® staining (Thermo Fisher Scientific, Waltham, MA, USA). Assays were analyzed with a GloMax® microplate reader (Promega, Madison, WI, USA). Fold change and growth rate were calculated and GR50 [21] was determined through a non-linear regression analysis. Statistical analysis was performed using GraphPad® software (2017, GraphPad Inc., California, USA)

Flow Cytometry assessment of proliferation rate

MIA PaCa-2 cells were pre-cultured at 5 % CO_2, Continuous 10 % CO_2 or intermittent exposure to 10 % CO_2 (3 hrs/day). Cells were labelled with CellTrace™ Violet fluorescent dye (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturer recommendations and left to grow in their respective conditions for 5 days. Cells were then collected and assessed using a BD FACS Celesta™ Flow cytometer (Becton, Dickinson and Company [BD], Warwick, Rhode Island, USA) using an excitation/emission of 405nm/450nm. Data were analysed using the FlowJO software (FlowJo LLC, Ashland, OR, USA).

Colony formation assays

Cells were seeded in 6-well plates and exposed to increased doses of ionizing radiation. Cells were washed with PBS and fixed with 80 % methanol solution. The cells were then stained with 0.5 % Crystal Violet solution (in 20 % methanol), washed with deionized water and left to air dry. Individual wells were assessed for colony count, surface area coverage and overall staining intensity using ImageJ (Version 1.52P, NIH, USA). Daily assessment experiments were performed, in which at the 4th day post seeding, the plates were exposed to increasing doses of radiation and singles wells were analysed in a daily regimen to

interrogate the time-dependent dynamics of the radiation effects. Parameters were normalized to the first day of collection (5th day).

Clinical data analyses

The clinical study was approved by the Thomas Jefferson University Hospital Institutional Review Board. Clinical data from an institutional database were used to retrospectively identify PDAC patients who had undergone pancreatic resection in the years 2002–2014. Self-reported current smokers were excluded to remove possible pro-tumorigenic influences from active cigarette smoking. Demographic data, comorbidities, pathologic findings and follow-up end points were recorded.

Statistical Analyses

Categorical data were compared by $\chi 2$ test or Fisher's exact test. Continuous variables were assessed for normality of distribution with the Kolmogorov-Smirnov test. Normally distributed continuous variables were compared using the Student's T-test. In cases of non-normal distribution patterns, comparisons between groups were performed using the Mann-Whitney test. Kaplan-Meier analysis (with log-rank comparisons) was used to compare between different survival risk factors for overall survival (OS) and disease-free survival (DFS). Factors with P value (<0.1) were subsequently included in a Cox multivariate hazard model. P values (≤0.05) were considered statistically significant. Statistical analyses were performed using the Statistical Package for Social Sciences (IBM SPSS, Ver.20, SPSS Inc., Chicago, IL, USA).

RESULTS

Cohort Description

An initial patient set composed of 1530 patients which underwent pancreatic operations at the Thomas Jefferson University Hospital was created. The cases were then selected for PDAC, and curative-intent surgery resulting in a set of 599 patients (52 % males). The median age was 68.9 years (IQR 60.7–76.8 years). Smoking history was positive in 254 patients (42.4 %) of the study cohort and 48 patients (8 %) in the cohort had a history of obstructive respiratory disease (asthma, chronic bronchitis or emphysema). Twenty-one patients (3.5 %) had missing data or unclear status regarding ORD and were excluded from further analysis (final cohort = 578 patients, as seen in Figure 1). The ORD group had significantly more female patients (62.5 % vs. 46.2 %, P<0.05) and higher rates of history of tobacco use (66.7 % vs. 39.6 %, P<0.01). A detailed description of the final cohort and comparison between study arms is shown in Table 1.

Patient data: Obstructive respiratory diseases are associated with decreased survival

The median overall survival duration of the entire cohort (N=578) was 18.9±0.9 months. T stage, N stage and tumor margin positivity were found to be significant risk factors (P<0.01) for mortality. Patients with obstructive respiratory disease had a significantly reduced OS (14.9±2.3 months vs. 19.8±1.1 months, P<0.05, Figure 2A). Disease recurrence data were missing in 60 patients (10.3%). The median disease-free survival for the evaulable cohort (N=518) was 22.2±1.2 months. T staging was not found to correlate with DFS. N staging

and tumor margin positivity were found to be significant risk factors for DFS (P<0.01). Patients in the ORD group had a shorter duration of DFS (13.9±2.2 months vs. 22.7±1.1 months, P=0.055, Figure 2B), however this finding was only borderline significant. Multivariate Cox survival regression analysis revealed ORD to be an independent risk factor for OS (HR 1.64, 95 % CI 1.18–2.3, P<0.01) and DSF (HR 1.68, 95 % CI 1.06–2.67, P<0.05) as shown in figures 2C and 2D, respectively.

Hypercapnic microenvironment promotes cell proliferation

Exposure of MIA-PaCa2 cells to 10 % CO_2 resulted in a significant increase in proliferation rate (replications/day) for both the continuous and intermittent exposure arms as compared to the 5 % CO_2 control arm (49.3 % ±8.2 % and 111.3 % ±4.7 %, respectively P<0.05). Exposure of PANC-1 cells to 10 % CO_2 resulted in a significant increase in proliferation rate only for the intermittent exposure arm as compared to the 5% CO_2 controls (114.3 % ±8.2 %, respectively, P<0.05) as seen in Figures 3A–B. Flow cytometry analysis showed a significant decrease in established (older) parental cells and a marked increase in newer generations (Figure 3C–E).

Hypercapnic microenvironment promotes radioresistance

MIA-PaCa2 cells were exposed to increasing doses of ionizing radiation as assessed in clonogenic assays. Intermittent exposure to 10 % CO_2 resulted in significantly greater colony surface area coverage as compared with 5 % CO_2 in non-irradiated cells and in the 2 Gy groups (fold change 1.44±0.06 and 1.28±0.07, respectively. P<0.05, Figure 4A). Of note, in cells treated with 4 Gy of radiation, 10 % CO_2 exposure resulted in a non-significant increase in colony surface area (fold change 1.1±0.1, P=NS). Daily assessment of colony formation post-radiation treatment revealed increased growth rates for cells exposed to 10 % CO_2 as compared with 5 % CO_2 controls after treatment with radiation (Figure 4B).

Hypercapnic microenvironment promotes chemoresistance

MIA-PaCa2 and PANC-1 cells were assessed for chemoresistance after exposure to increasing amounts of clinically relevant DNA damaging therapeutics (i.e., gemcitabine and oxaliplatin) cultured under normal or elevated CO_2 (continuous and intermittent 10 % CO_2), as shown in Figure 5. In the MIA-PaCa2 cells, GR50 values for oxaliplatin indicated increased resistance upon exposure to hypercapnic conditions (continuous: 2.47 μM and intermittent: 3.8μM vs. controls:1.53 μM, P<0.05). GR50 values for gemcitabine did not show significant change upon exposure to hypercapnic conditions as compared with 5 % CO_2 exposure. However, cells exposed to intermittent 10 % CO_2 displayed greater susceptibility to a high dose of gemcitabine (P<0.05). In PANC-1 cells, GR50 values for oxaliplatin indicated increased resistance upon exposure to continuous 10 % hypercapnic conditions compared with 5 % CO_2 controls (10.42 μM vs. 3.03 μM, P<0.05). Intermittent exposure to 10 % CO_2 resulted in a non-significant increase in GR50 for oxaliplatin as compared with 5 % CO_2 controls (3.74 μM vs. 3.03 μM, P=NS).

DISCUSSION

Smoking is an established major risk factor for the development of PDAC, which can account for nearly a 2.4-fold increase in PDAC risk in a dose-dependent manner [22, 23]. Other respiratory diseases associated with hypoxia and hypercapnia, such as COPD and obstructive sleep apnea are associated with an increased risk of cancer [12–15, 19]. While tumor hypoxia has been repeatedly shown to be a critical factor in PDAC therapy resistance [24–27], only a few studies have attempted to look at hypercapnia as a cancer resistance promoting factor. For example, Obata et al [9] have shown that transient hypercarbia increases cell invasion in colon cancer cells, possibly through an upregulation of matrix metallopeptidase 2 and 9 (MMP2/MMP9) protein expression. Moreover, abdominal insufflation of 100 % CO_2 was shown to promote cell proliferation [28] and cell invasion [29] in mouse colon cancer models, *in vivo*.

In this study we have chosen to investigate whether COPD and asthma, commonly associated with hypercapnia, have a prognostic role in PDAC survival. We included only non-smokers or past smokers in our analysis, thereby excluding possible confounding effects from active smoking or inhaled chemicals (e.g, nicotine[30–33]). Our results indicate that ORD are associated with increased mortality risk and shorter disease-free survival. We have then further assessed, *in vitro*, the different phenotypic changes that occur in PDAC cells in the presence of hypercapnia. We have found that intermittent and continuous exposure to 10 % CO_2 induced increased proliferation and resistance to radiotherapy. Furthermore, such exposures resulted in relative resistance to oxaliplatin therapy, as seen in Figure 5.

Concordant with our findings, Kikuchi et al [5] have described a non-pH dependent resistance to reactive oxygen species stress and to platinum-based chemotherapies in lung cancer cells exposed to hypercapnic conditions (*e.g.,* 15 % CO_2). These data support our clinical findings that show increased disease progression (i.e., a worse prognosis) in patients that have increased CO_2 retention, as is common in COPD and severe asthma patients. Overall, our study reveals hypercapnia to be another disease modifier in PDAC patients with ORD, distinct from hypoxia, but sharing some common phenotypes. These findings may be clinically relevant as they offer a potential personalized approach for ORD patients. In fact, we propose a future prospective clinical trial to optimize ventilatory function as part of patient pre-habilitation prior to oncologic treatment. The interventions would be aimed at reducing the CO_2 load through improving ventilation with pharmacologic agents, intensive respiratory therapy, and/or dietary modifications aimed to adjust the respiratory quotient (RQ)[34–36].

This study has several limitations which merit discussion. Clinically, the study is based on a single center, retrospective design, limiting its generalizability and interpretation. Smoking history data were based on self-reports and thus are prone to bias. Additionally, ORD status was determined based upon data in the electronic medical records, rendering the data open for potential misclassification. As noted, hypoxia is a known promoter of cancer resistance and its occurrence in ORD may serve as a confounding factor in the interpretation of the survival data. Our study did not control for additional non-cigarette smoke harmful particulates (e.g., occupational exposures). Other possible confounders such as gender and

age were also identified. However, these were included in our regression models to account for their effects. It is important to note that while our *in vitro* data corroborates our clinical findings, we have yet to validate our results in an *in vivo* model.

CONCLUSIONS

Hypercapnic conditions promote *in vitro* pancreatic cancer cell proliferation and resistance. Patients with PDAC who have obstructive respiratory disorders associated with CO_2 retention have increased mortality and shorter disease-free survival. These findings support the need for a clinical trial that may set the stage for a personalized therapeutic approach for patients with ORD and PDAC.

Acknowledgments

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Abbreviations and Acronyms

DFS	disease-free survival
GR_{50}	growth rate inhibition
ORD	obstructive respiratory disease
OS	overall survival
PDAC	pancreatic ductal adenocarcinoma

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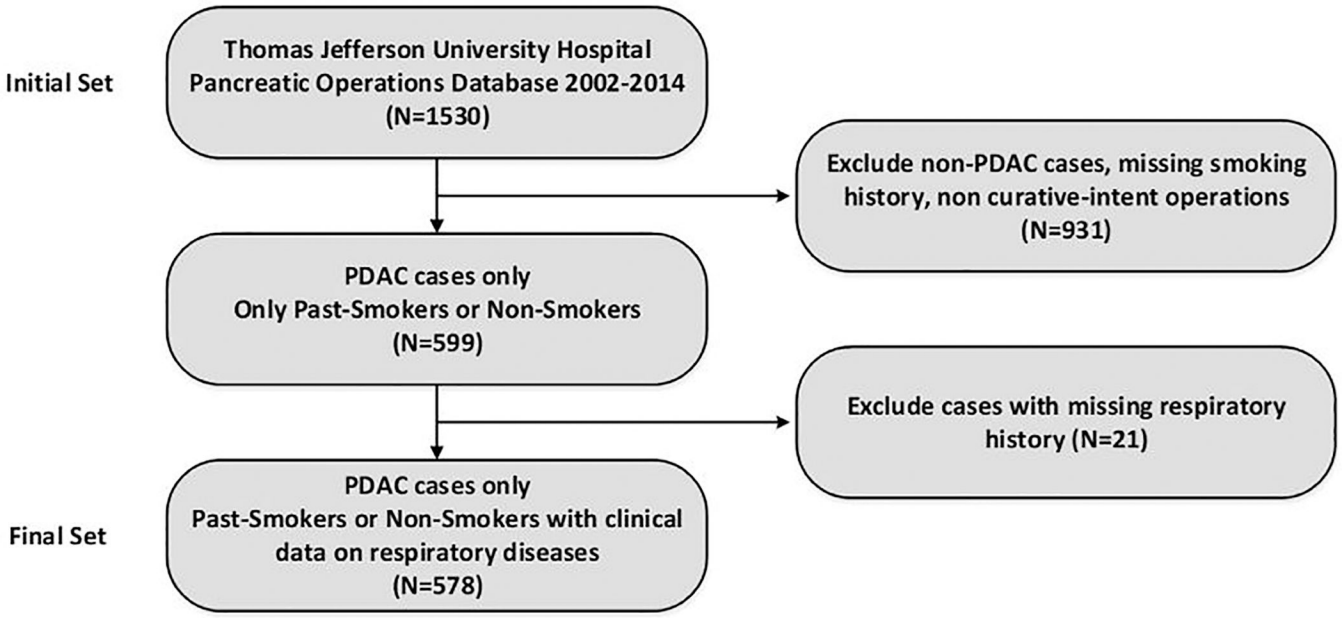

Figure 1.
Cohort selection flow diagram. PDAC, pancreatic ductal adenocarcinoma

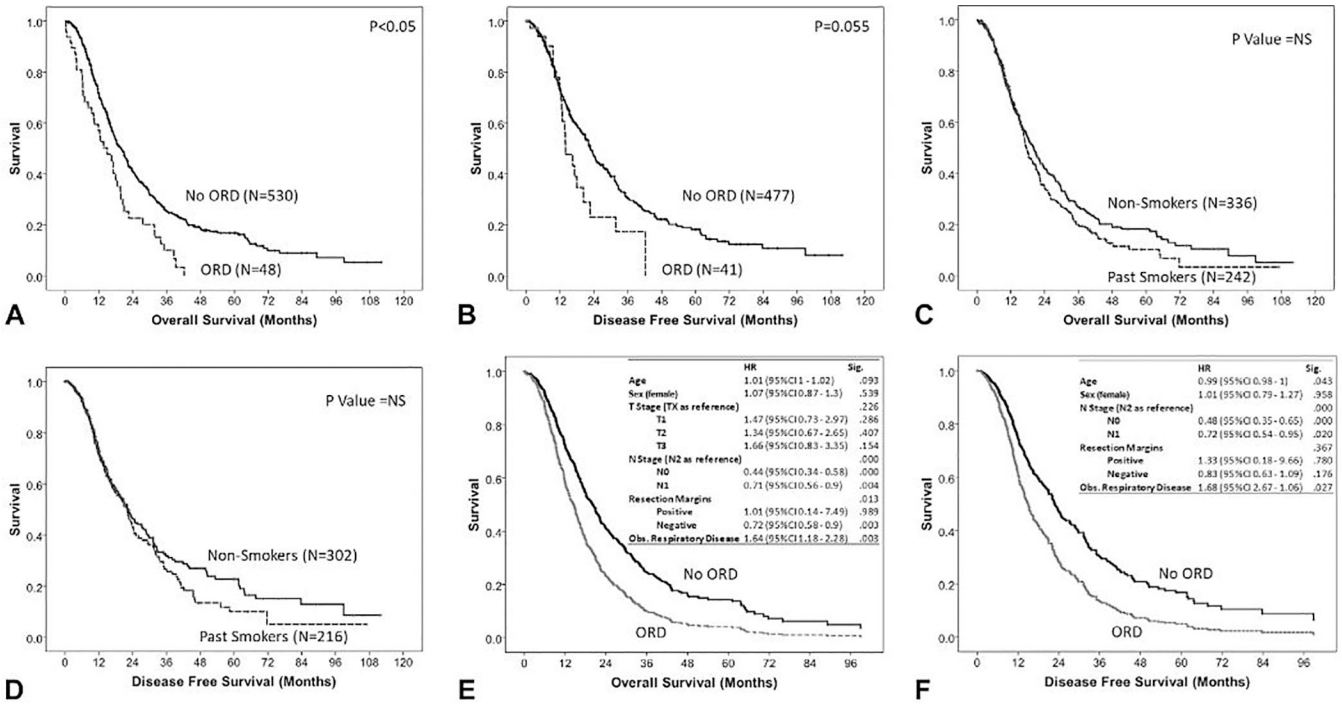

Figure 2.

Thomas Jefferson University cohort (N=578) survival analysis showing Kaplan-Meier plots comparing obstructive respiratory disorders (ORD) and non-ORD groups for (A) overall survival and (B) disease-free survival; Kaplan-Meier plots comparing smoking history groups for (C) overall survival and (D) disease-free survival; and Cox regression models for (E) overall survival and (F) disease-free survival. HR, hazard ratio; NS, not significant

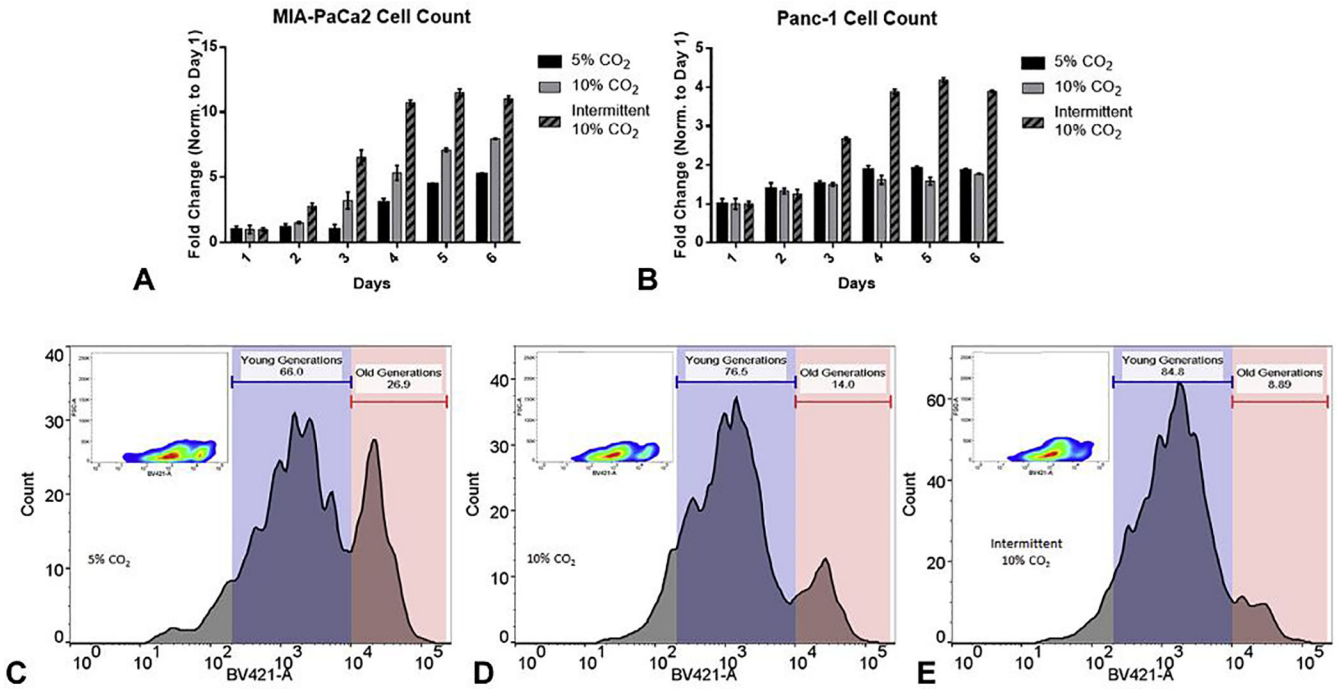

Figure 3.

Proliferation assays. daily quantification based on dsDNA measurement in (A) MIA-Paca2 and (B) Panc-1 pancreatic cancer cell lines exposed to intermittent or continuous 10% CO_2 compared to control conditions of 5% CO_2 (Mean ±SEM). (C-E) Flow cytometry of labeled MIA-Paca2 cells after long-term exposure to intermittent or continuous 10% CO_2 compared to 5% CO_2 controls ($p<0.05$)

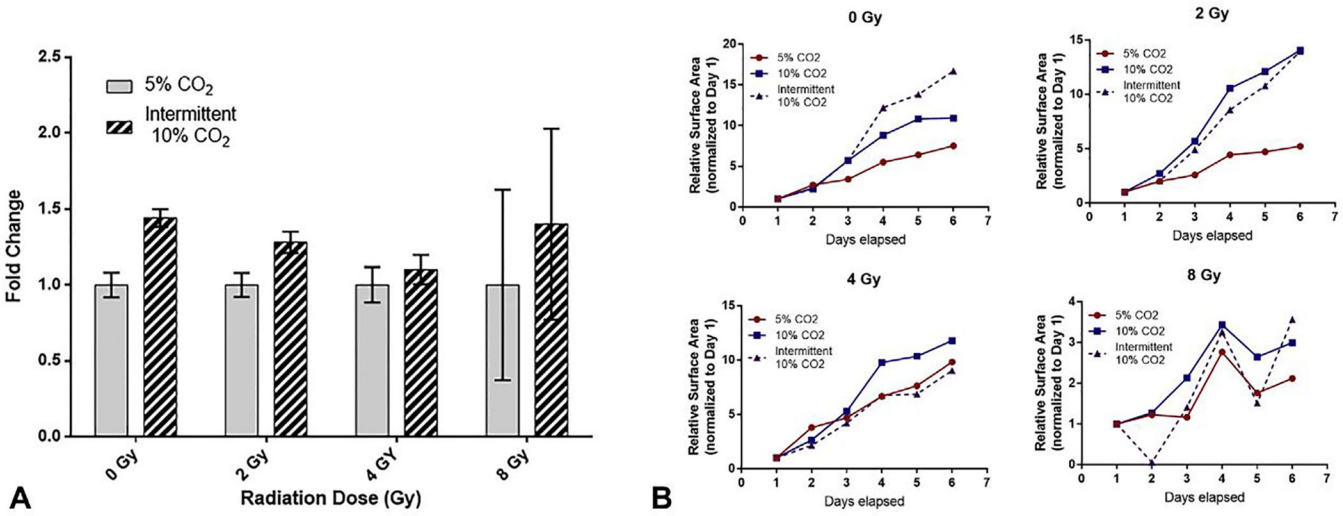

Figure 4.

Radiotoxicity clonogenic assays in MIA-Paca2 cancer cells seeded in 6-well plates. (A) Surface area measurement comparison between intermittent exposure to 10% CO_2 and 5% CO_2 controls (Mean ±SEM). Statistical significance ($P<0.05$) noted at 0 Gy and 2 Gy of radiation dose. (B) Normalized daily growth trends in response to ionizing radiation comparing 10% CO_2 intermittent, 10% CO_2 continuous and 5% CO_2 continuous exposures.

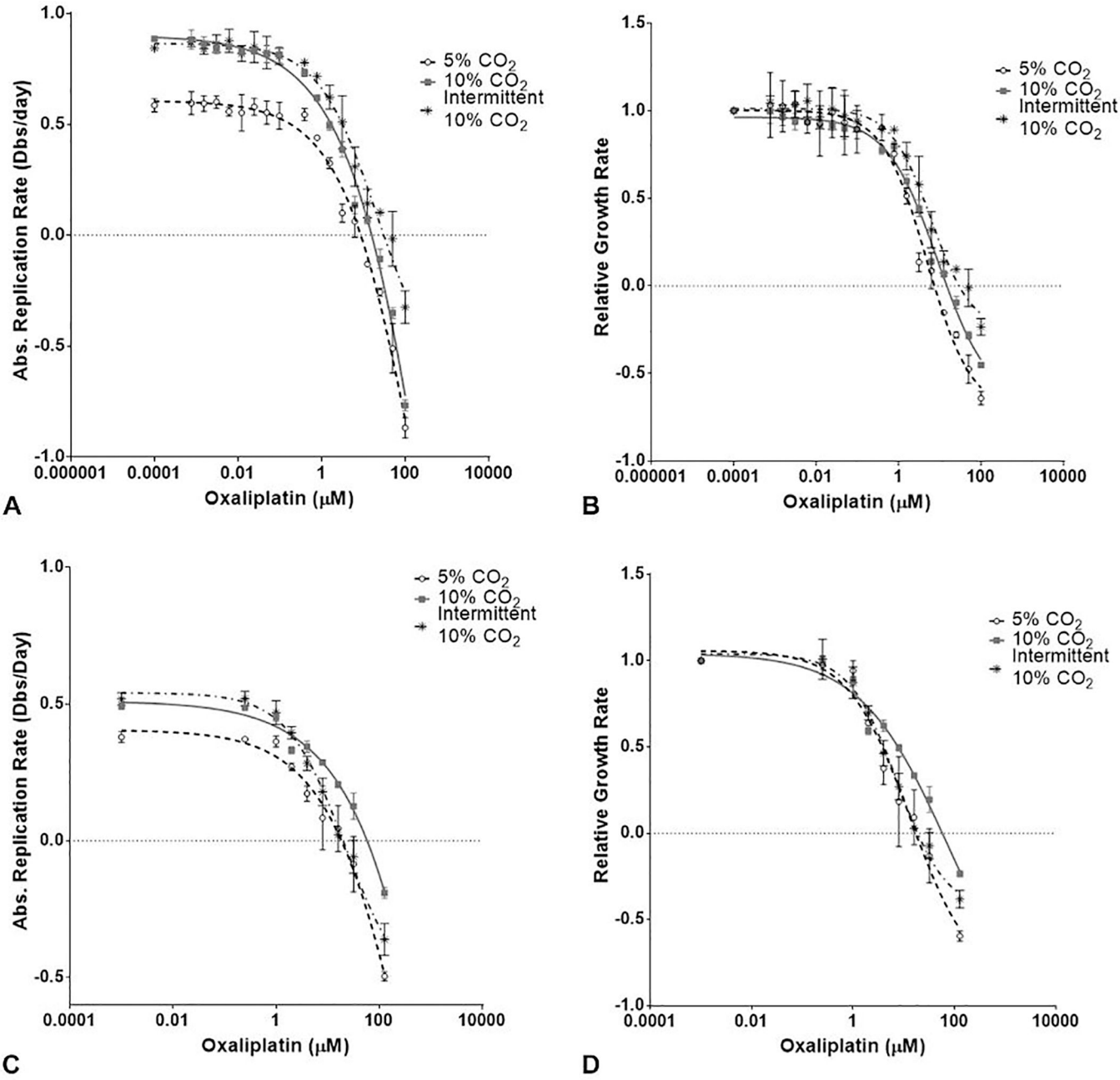

Figure 5.

Oxaliplatin response in cell viability assays absolute replication rate and GR50 in MIA-PaCa 2 cells (A,B) and Panc-1 cells (C,D).

Table 1.

Cohort Characteristics (n = 578)

Characteristic	Overall (n = 578)	No obstructive respiratory disease (n = 530)	Obstructive respiratory disease (n = 48)	p Value*
Age, y, median (IQR)	68.7 (60.6–76.8)	68.5 (60.1–76.9)	70.6 (64.3–76.8)	NS (0.1)
Sex, n (%)				0.033
Male	303 (52.4)	285 (53.8)	18 (37.5)	
Female	275 (47.6)	245 (46.2)	30 (62.5)	
Race, n (%)				NS
African American	44 (7.6)	43 (8.1)	1 (2.1)	
Asian	17 (2.9)	17 (3.2)	0 (0)	
White	503 (87.0)	456 (86)	47 (97.9)	
Other	2 (0.3)	2 (0.4)	0 (0)	
Unknown	12 (2.1)	12 (2.3)	0 (0)	
Smoking history, n (%)				0.001
Nonsmoker	336 (58.1)	320 (60.4)	16 (33.3)	
Past smoker	242 (41.9)	210 (39.6)	32 (66.7)	
Obstructive respiratory disease, n (%)				—
No lung disease	530 (88.5)	—	—	
COPD	29 (4.8)	—	—	
Asthma	19 (3.2)	—	—	
Unknown	21 (3.5)	—	—	
Tumor staging				
T				NS
T1	107 (18.5)	101 (19.1)	6 (12.5)	
T2	317 (54.8)	288 (54.3)	29 (60.4)	
T3	138 (23.9)	125 (23.6)	13 (27.1)	
TX	16 (2.8)	16 (3)	0 (0)	
N				NS
N0	191 (33)	171 (32.3)	20 (41.7)	
N1	212 (36.7)	202 (38.1)	10 (20.8)	
N2	175 (30.0)	157 (29.6)	18 (37.5)	

IQR, interquartile range.

*A p value < 0.05 is considered statistically significant.