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Continuous exposure of pancreatic cancer cells to dietary bioactive agents does not induce drug resistance unlike chemotherapy

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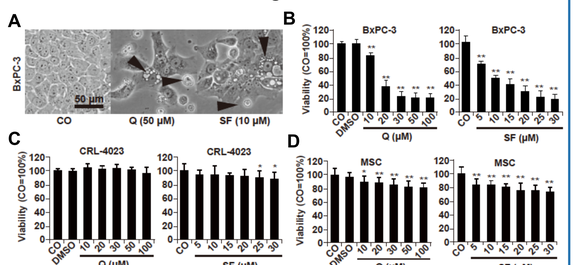
Abstract

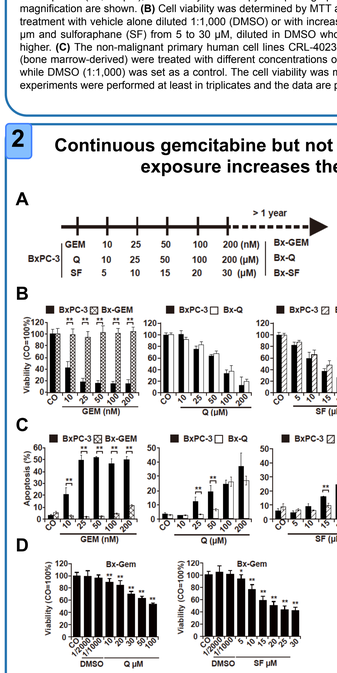
The repeated treatment of cancer cells with chemo- or radiotherapy induces therapy resistance, but it was previously unknown whether the same effect occurs upon continuous exposure of cancer cells to diet-derived chemopreventive agents. We elucidated this interesting question in pancreatic ductal adenocarcinoma, which is a highly aggressive cancer entity with a marked resistance toward gemcitabine and other cytotoxic drugs. The isothiocyanate sulforaphane (SF), present in cruciferous vegetables, and the polyphenol quercetin (Q), present in many fruits and vegetables induced apoptosis and reduced viability in gemcitabine (GEM)-sensitive BxPC-3 cells but not in non-malignant ductal pancreas cells and mesenchymal stromal cells. In turn, BxPC-3 cells were incubated treated with increasing concentrations of GEM, SF or Q for more than one year and the surviving subclones Bx-GEM, Bx-SF and Bx-Q were selected, respectively. Whereas Bx-GEM cells acquired a total resistance, Bx-SF or Bx-Q cells largely kept their sensitivity as proved by MTT assay, Annexin V staining and FACS-analysis. The evaluation of the self-renewal-, differentiation- and migration potential by colony formation, differentiation, or migration assays demonstrated that cancer stem cell features were enriched in GEM-resistant cells, but decreased in SF- and Q-long time-treated cells. These results were confirmed by orthotopic xenotransplantation of cancer cells to the mouse pancreas, where Bx-GEM formed large, Bx-Q small and Bx-SF cells almost undetectable tumors. An mRNA expression profiling array and subsequent gene set enrichment analysis and qRT-PCR confirmed that tumor progression markers were enriched in Bx-GEM, but reduced in Bx-SF and Bx-Q cells.

1 Quercetin and sulforaphane selectively reduce the viability in malignant cells



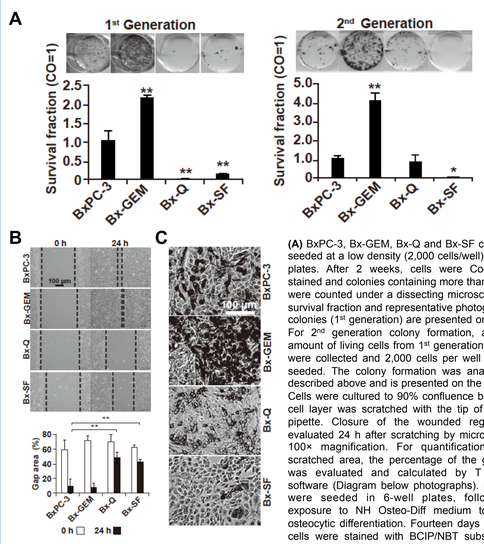
(A) The human PDA cell line BxPC-3 was left untreated (CO) or was treated with quercetin (Q, 50 µM) or sulforaphane (SF, 10 µM), followed by microscopy and photography 48 hours later. Representative pictures at 200× magnification are shown. **(B)** Cell viability was determined by MTT assay in untreated BxPC-3 cells (CO) or 72 h after treatment with vehicle alone diluted 1:1,000 (DMSO) or with increasing concentrations of quercetin (Q) from 5 to 100 µm and sulforaphane (SF) from 5 to 30 µM, diluted in DMSO whose final concentration in medium was 1:1,000 or higher. **(C)** The non-malignant primary human cell lines CRL-4023 (immortalized ductal pancreas cells) or **(D)** MSC (bone marrow-derived) were treated with different concentrations of quercetin (Q) or sulforaphane (SF) as indicated, while DMSO (1:1,000) was set as a control. The cell viability was measured as described above. Three independent experiments were performed at least in triplicates and the data are presented as means ±SD.

2 Continuous gemcitabine but not sulforaphane or quercetin exposure increases therapy resistance



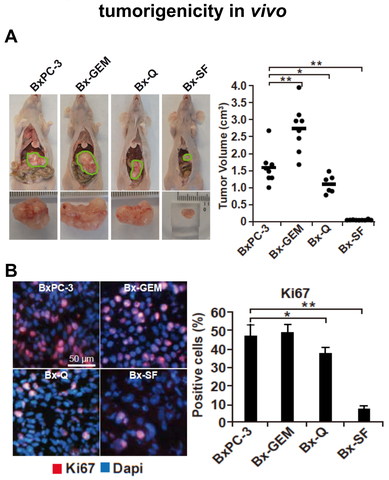
(A) BxPC-3 cells were treated with gemcitabine (GEM, 10 nM), quercetin (Q, 10 µM) or sulforaphane (SF, 5 µM) at a confluence of 40-60%. After two weeks, when the cells recovered, they were treated again with higher concentrations of each agent as indicated, followed by a recovery phase and a new round of treatment with higher concentrations. After more than one year of repeated treatment with gradually higher concentrations, the highest concentrations of 200 nM GEM, 200 µM Q and 30 µM SF were reached and the resulting final subclones were named Bx-GEM, Bx-Q and Bx-SF, respectively. These final subclones were used for all following experiments. **(B)** The cells were treated with different concentrations of gemcitabine, quercetin or sulforaphane as indicated. Seventy-two hours later, the viability was measured by MTT assay. **(C)** Likewise, the percentage of apoptotic cells was measured by staining with Annexin V-FITC and PI followed by FACS-analysis. **(D)** Bx-GEM cells were treated with quercetin or sulforaphane at concentrations indicated and 72 h later the viability was measured by MTT assay. Three independent experiments were performed for Fig. 2B-D (MTT assay n=8, apoptosis assay n=3) and the data are presented as means ±SD.

3 Continuous quercetin and sulforaphane but not gemcitabine exposure reduces tumorigenicity in *vitro*



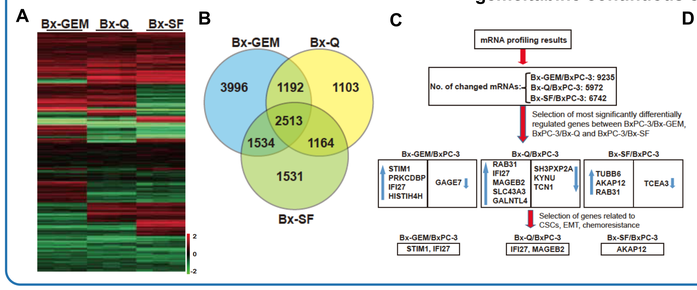
(A) BxPC-3, Bx-GEM, Bx-Q and Bx-SF cells were seeded at a low density (2,000 cells/well) in 6-well plates. After 2 weeks, cells were Coomassie-stained and colonies containing more than 50 cells were counted under a dissecting microscope. The survival fraction and representative photographs of colonies (1st generation) are presented on the left. For 2nd generation colony formation, an equal amount of living cells from 1st generation colonies were collected and 2,000 cells per well were re-seeded. The colony formation was analyzed as described above and is presented on the right. **(B)** Cells were cultured to 90% confluence before the cell layer was scratched with the tip of a 10-µL pipette. Closure of the wounded region was evaluated 24 h after scratching by microscopy at 100× magnification. For quantification of the scratched area, the percentage of the gap area was evaluated and calculated by T Scratch software (Diagram below photographs). **(C)** Cells were seeded in 6-well plates, followed by exposure to NH Osteo-Diff medium to induce osteocytic differentiation. Fourteen days later, the cells were stained with BCIP/NBT substrate for alkaline phosphatases, expressed by cells differentiated to osteocytes, which appear dark. Representative images at 200× magnification are shown. **(D)** Proteins were harvested and the expression of EpCAM, Nanog, Twist2 and E-cadherin was measured by Western blot analysis. β-actin was used as a loading control. Three independent experiments were performed at least in triplicates and the data are presented as means ±SD.

4 Continuous exposure to quercetin or sulforaphane but not gemcitabine reduces tumorigenicity in *vivo*



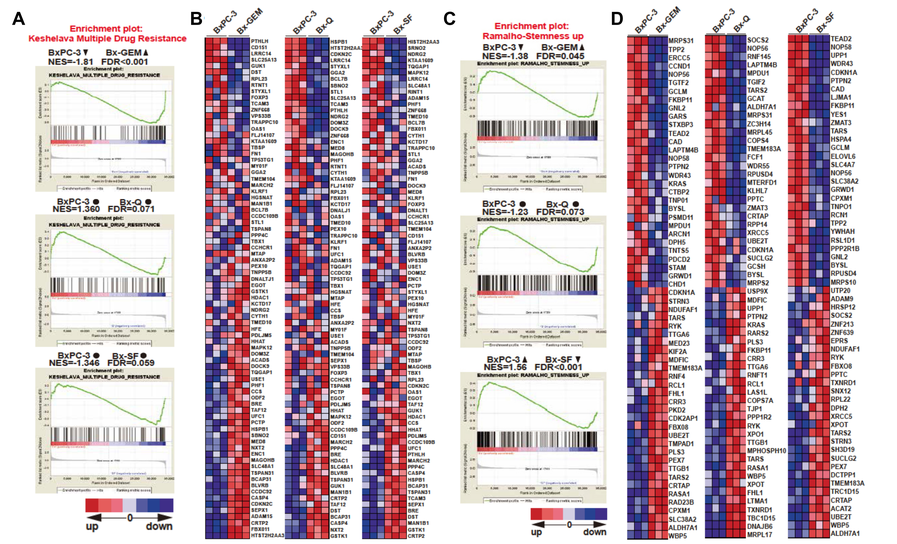
(A) Immunodeficient mice (n=8/per cell line) were anesthetized, followed by surgical intervention to expose the pancreas and to inject 1×10⁶ cells in 10 µL matrigel into the pancreatic head. After closure of the wound, the mice were kept for 6 weeks to allow tumor development, followed by euthanasia, tumor resection and measurement of tumor volumes by calipers. Representative images of tumor xenografts from each group are shown on the left and a diagram with the individual tumor volumes and the means of each group ±SD on the right. **(B)** Tumor tissue sections from xenografts were evaluated by immunofluorescence staining for the expression of the proliferation marker Ki67 and representative pictures at 200× magnification are shown on the left. The percentage of positive cells was counted and the means ±SD are shown in the diagram on the left.

5 Continuous quercetin and sulforaphane exposure reduces gene array-analyzed expression of progression markers but not in gemcitabine continuous exposure



(A) mRNA was harvested from BxPC-3, Bx-GEM, Bx-Q and Bx-SF cells followed by gene array analysis and biostatistical evaluation of statistically significant expression patterns between the different cell lines. The heat map shows the relative expression of differentially regulated genes in Bx-GEM, Bx-Q and Bx-SF cells compared to parental BxPC-3 cells. **(B)** The numbers of differentially regulated genes in Bx-GEM, Bx-Q and Bx-SF cells compared to parental BxPC-3 cells and compared between the groups are presented in a Venn-diagram ($p<0.01$). **(C)** Flow diagram of the computational selection process of miRNA candidates from the miRNA profiling results shown above. Upward blue arrows: up-regulation of mRNAs compared to BxPC-3; Downward blue arrows: down-regulation of mRNAs compared to BxPC-3. **(D)** RNA was harvested from BxPC-3, Bx-GEM, Bx-Q and Bx-SF cells and mRNA expression of IFI27, STIM1, MAGEB2 and AKAP12 was analyzed by qRT-PCR. The expression in BxPC-3 cells was set to 1. GAPDH was used as endogenous control. The qRT-PCR was performed in triplicates three times with similar outcome and the means ±SD are shown.

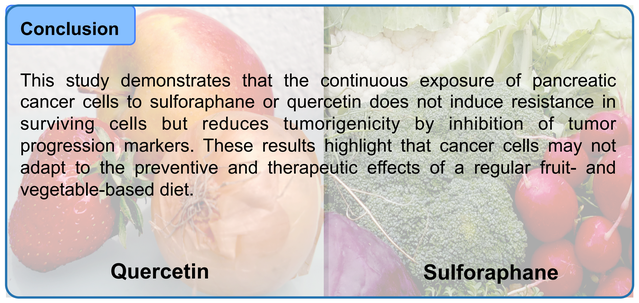
6 Continuous quercetin and sulforaphane exposure reduces gene set enrichment-analyzed multidrug resistance and stemness genes but not in gemcitabine continuous exposure



(A) Significantly differentially regulated genes from the gene array analysis described in Fig. 5 were further analyzed by a gene set enrichment analysis with an available set of genes known to be involved in multidrug resistance (KESHELAVA_MULTIPLE_DRUG_RESISTANCE), NES: normalized enrichment score. FDR: false discovery rate. Enriched. No change. Decreased. The differences were considered significant if FDR values were less than 0.05. The detailed process is described in the Materials & Methods section. **(B)** Heat map of the differentially regulated mRNAs according to gene set enrichment analysis as described in point A. The scale marks the relative changes of differentially regulated mRNAs in BxPC-3, Bx-GEM, Bx-Q and Bx-SF: up regulated (red), no change (gray) and down regulated (blue). **(C)** Significantly differentially regulated genes from the gene array analysis described in Fig. 5 were further analyzed by a gene set enrichment analysis with an available set of genes known to be involved in multidrug resistance (KESHELAVA_MULTIPLE_DRUG_RESISTANCE). NES: normalized enrichment score. FDR: false discovery rate. Enriched. No change. Decreased. The differences were considered significant if FDR values were less than 0.05. The detailed process is described in the Materials & Methods section. **(D)** Heat map of the differentially regulated mRNAs according to gene set enrichment analysis as described in point A. The scale marks the relative changes of differentially regulated mRNAs in BxPC-3, Bx-GEM, Bx-Q and Bx-SF: up regulated (red), no change (gray) and down regulated (blue).

Conclusion

This study demonstrates that the continuous exposure of pancreatic cancer cells to sulforaphane or quercetin does not induce resistance in surviving cells but reduces tumorigenicity by inhibition of tumor progression markers. These results highlight that cancer cells may not adapt to the preventive and therapeutic effects of a regular fruit- and vegetable-based diet.



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