Heparanase induces epithelial mesenchymal transition in gastric signet-ring cell adenocarcinoma

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Abstract:

Heparanase (HPSE), a heparan sulfate-specific endo-β-D-glucuronidase, plays an important role in tumor cell metastasis through the degradation of extracellular matrix heparan sulfate proteoglycans. Suramin, a polysulfonated naphthylurea, is an inhibitor of HPSE with suramin analogues. Our objective was to analyze the HPSE involvement in gastric signet ring cell adenocarcinoma (SRCA) invasion. High expression of HPSE mRNA and protein was found in the tumor and in ascites of SRCA as well as in KATO-III cell line. Beside of collagen-I, growth factors (TGF-β1 and VEGF-A, except FGF-2) and epithelial mesenchymal transition (EMT) markers (Snail, Slug, Vimentin, α-SMA and Fibronectin, except E-cadherin) were found higher in main nodules of SRCA as compared to peritumoral sites. Among MDR proteins, MDR-1 and LRP (lung resistance protein) were highly expressed in tumor cells. The formation of 3D cell spheroids was found to be correlated with their origin (adherent or non-adherent KATO-III). After treatment of KATO-III cells with a HPSE inhibitor (suramin), cell proliferation and EMT-related markers, besides collagen-1 expression, were down regulated. In conclusion, in SRCA, HPSE via an autocrine secretion is involved in acquisition of mesenchymal phenotype and tumor cell malignancy. Therefore, HPSE could be an interesting pharmacological target for the treatment of SRCA.

Keywords: Signet ring cell adenocarcinoma, KATO-III cell lines, Heparanase, Epithelial mesenchymal transition, Carcinomatosis

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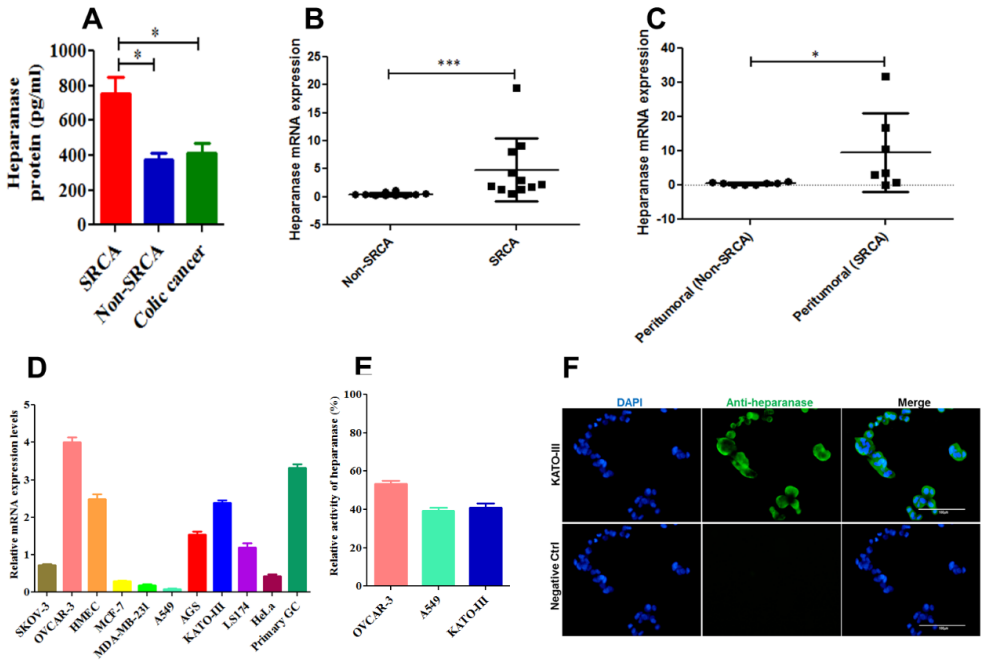
MATERIALS AND METHODS

Cell lines and reagents: Human cancer cell lines used, ovarian (OVCAR-3) and gastric (AGS, KATO-III) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). , Ascitic fluids from 14 cancer patients of the Hospital Lariboisière (Paris, France) were collected. As ascitic fluid evacuation is a part of routine management of patients, only oral consents were obtained from them. Cells from ascitic fluids were pelleted by a short spin at 1000 rpm and supernatants were collected after a 10 min centrifugation. Drug used in this study was suramin (Sigma Chemical Co, St. Louis, MO, USA).

Tissues : Tumor and corresponding normal gastric tissue specimen (SRCA tumoral, SRCA peritumoral, Non-SRCA tumoral and Non-SRCA peritumoral) were obtained from 21 patients with signet ring cell adenocarcinoma from the General and Digestive Tract Surgery Department at Lariboisière Hospital in Paris (France). Informed consent was obtained from each patient prior to surgery. All of the tumor and macroscopically normal gastric tissue samples were obtained at the time of surgery. These tissue samples were rapidly frozen in liquid nitrogen and stored at −80° C until analysis. Tissue samples were histologically confirmed by hematoxylin and eosin staining.

Human phosphokinase array : A membrane-based antibody array (R&D Systems, Raffles, China) that determines the relative levels of 45 different human phosphorylated protein kinases was used according to the manufacturer's instructions. Briefly, equal amounts of cell lysates of KATO-III cell line treated with or without 200 µM Suramin (Sigma Chemical Co, St. Louis, MO, USA) into FBS free IMDM medium along with control for 5 hrs were incubated overnight with the phosphokinase array membrane. The array was washed to remove unbound proteins followed by incubation with a mixture of biotinylated detection antibodies. Streptavidin- HRP and chemiluminescent detection reagents were applied to visualize the signal produced at each capture spot corresponding to the amount of phosphorylated protein bound with densitometry by using a photosensitive film (Kodak, X-OMAT, AR, USA).

Gastric signet ring cell adenocarcinoma nodules express HPSE



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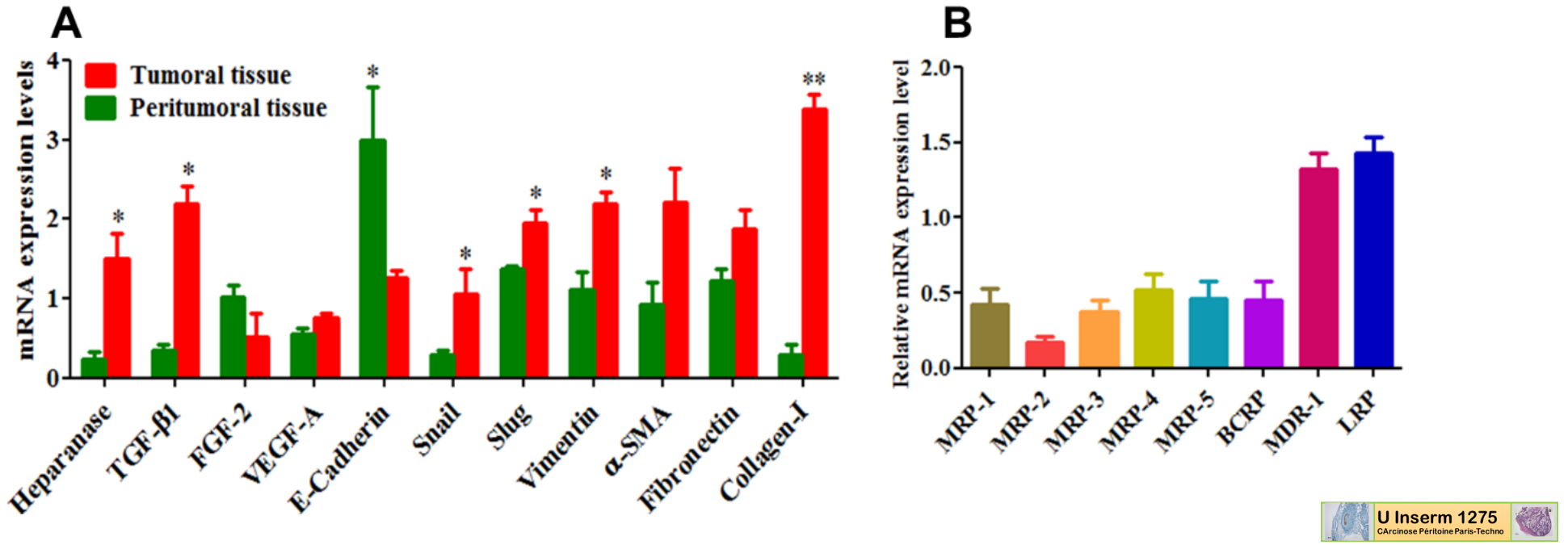
mRNA and protein expression of heparanase in clinical samples and cell lines including KATO-III

Heparanase protein was found in the ascitic samples of primary signet ring cell adenocarcinoma (SRCA) of stomach as compared to Non-SRCA of stomach and colic cancer by ELISA (SRCA n=5, Non-SRCA n=3 and colic cancer n=6) **(A)** mRNA expression of heparanase was found higher in SRCA (n=11) than non-SRCA (n=10) **(B)** as well as in peritumoral-SRCA (n=7) than peritumoral non-SRCA (n=8) **(C)** Heparanase gene expressed by various cell lines ovarian (OVCAR-3 and SKOV-3), breast (MDA-MB231 and MCF7), gastric (AGS, KATO-III), intestinal (LS174T), lung (A549), leukemia (K562), cervical (HELA), human microvascular endothelial (HMEC-1) cell lines and primary SRCA (Primary GC) via RT-PCR, **(D)** Heparanase activity (evaluated by degradation of fondaparinux at pH 5) observed in supernatants of various cancer cell lines (OVCAR-3), lung (A549) and gastric (KATO-III), **(E)** Heparanase protein expression level in KATO-III by immunofluorescence is shown **(F)** The results are expressed as mean ± SEM of three independent experiments *P<0.05, ***P<0.001, statistically significant.

Gastric signet ring cell adenocarcinoma express EMT and multi drug resistance markers



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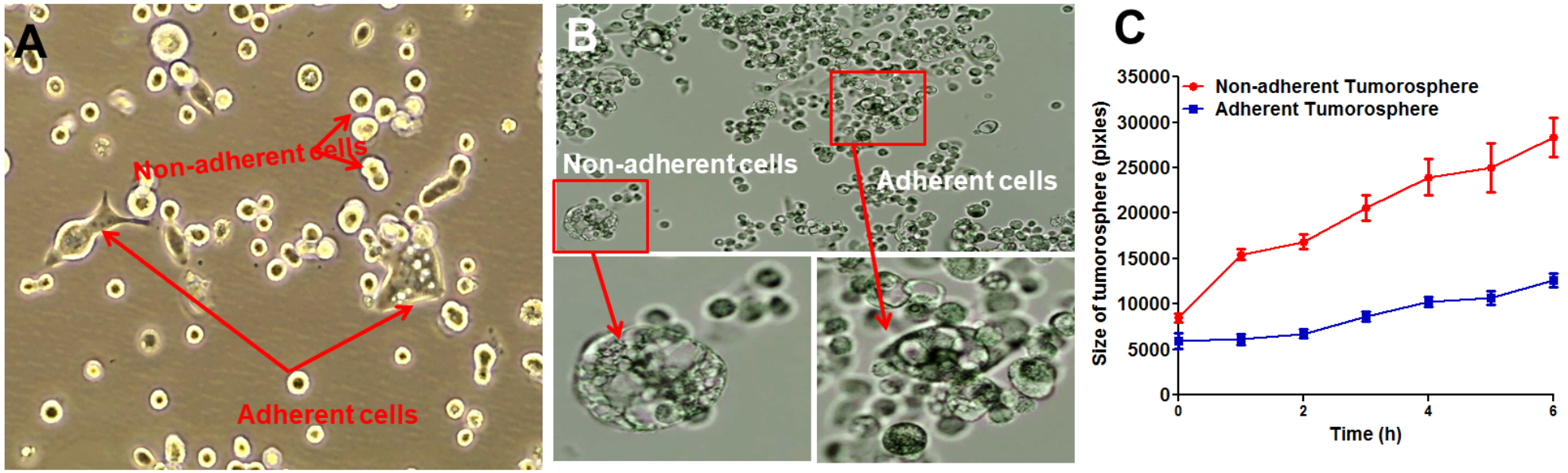
mRNA expression of heparanase, growth factors, EMT markers and drug transporters in clinical samples

Heparanase, growth factors (TGF-β1 and VEGF-A) except FGF-2 and epithelial marker like E-cadherin were found higher while mesenchymal markers (Snail, Slug, Vimentin, α-SMA and fibronectin) were lower in tumoral tissue of SRCA as compared to peritumoral tissue by qPCR **(A).** Similarly, of all the drug transporters (MDR-1, MDR-2, MDR-3, MDR-4, MDR-5, BCRP, MDR-1 and LRP) **o**nly two (MDR-1 and LRP) were found higher in SRCA tissue samples **(B).** The results are expressed as mean ± SEM of six independent experiments *P<0.05, **P<0.01, statistically significant.

KATO-III cell line formed spheroid clusters and expressed EMT markers *in vitro*



Spheroid cluster formation as well as cytokine array of both adherent and non-adherent KATO-III cell line.

Adherent and non-adherent cells of KATOIII **(A)** Tumorosphere cells derived from adherent and non-adherent KATO-III **(B)** quantification of the maximal tumorosphere outgrowth diameter (pixels) during 6hrs **(C).**

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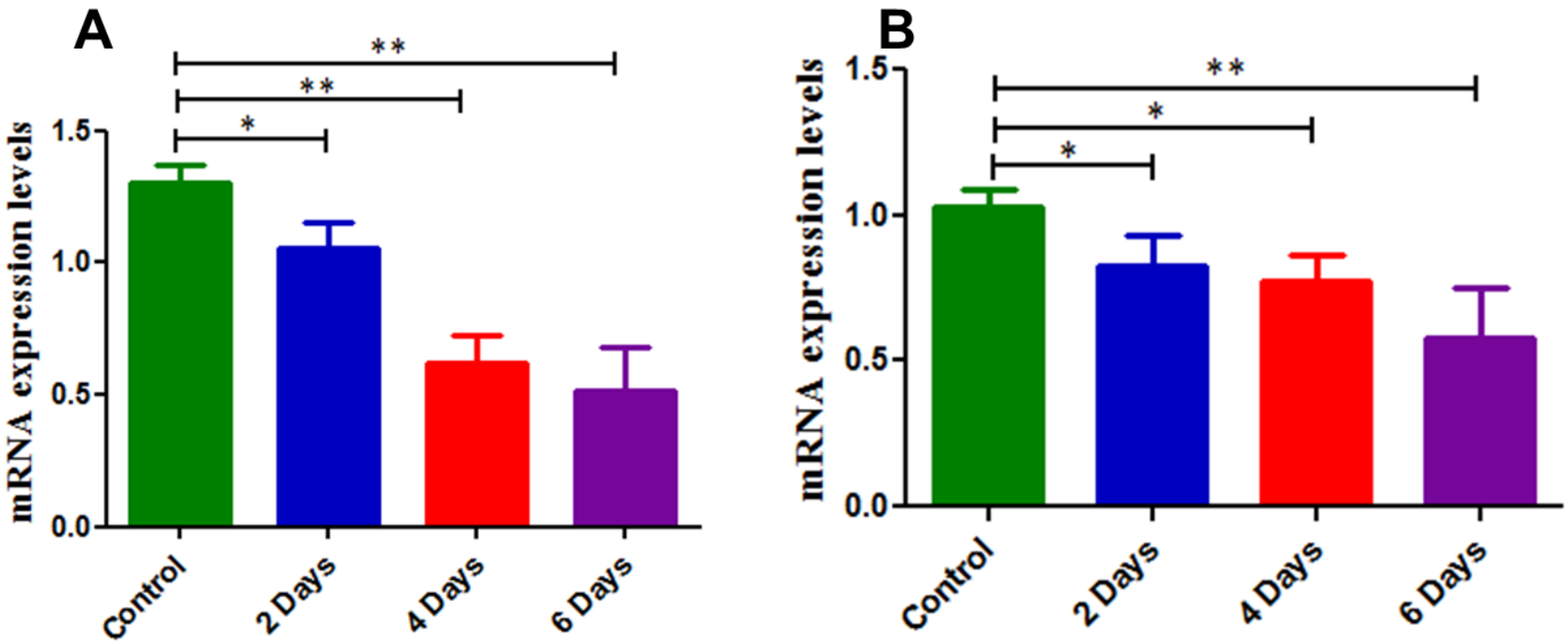
Suramin, an HPSE inhibitor down regulated TGF-β and collagen-1 mRNA KATO-III cells



mRNA gene expression of TGFβ-1 and collagen-I in KATO-III cell line after treatment with suramin.

Suramin lowers the expression of TGFβ-1 **(A)** and collagen-I **(B)** in KATO-III in a time dependent manner by qPCR. The results are expressed as mean ± SEM of six independent experiments *P<0.05, **P<0.01, statistically significant

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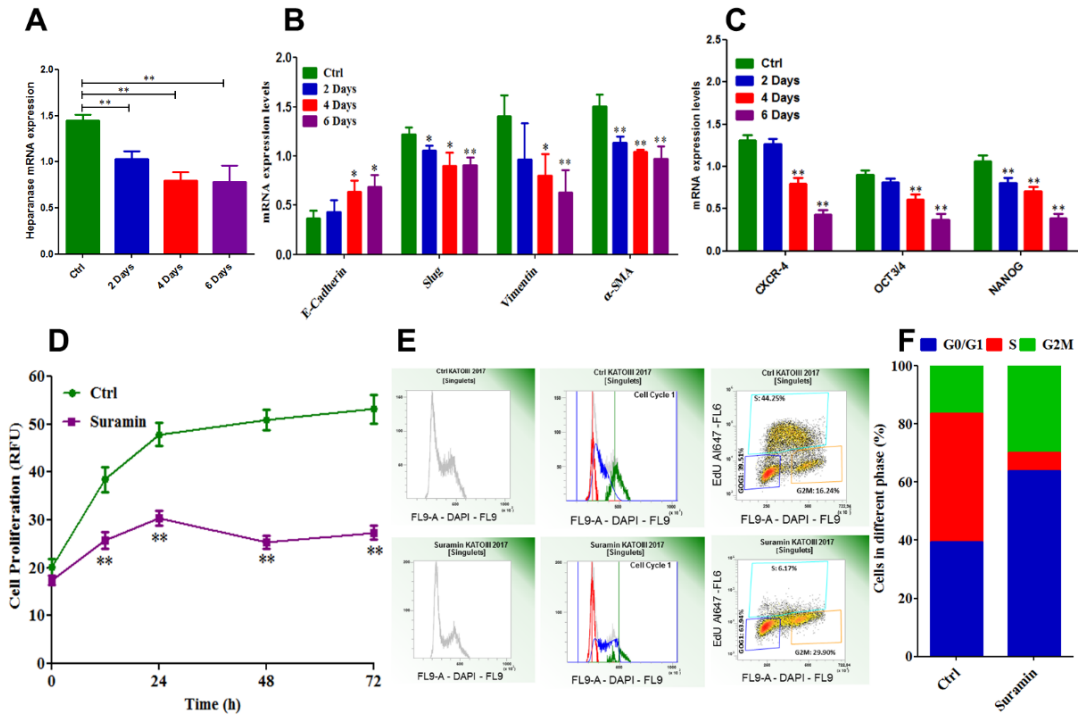
Suramin down regulates EMT and stem cell markers as well as inhibits cell cycle and proliferation of KATO-III cell line



mRNA expression of heparanase and EMT markers via qPCR, stem cell markers by flow cytometry, cell proliferation and coloration of differentiated KATO-III cells after induction

Expression of heparanase **(A)** and mesenchymal markers (Slug, Vimentin and α-SMA) were found lower while the epithelial marker (E-cadherin) was found higher **(B)** in KATO-III by qPCR after inducing differentiation. Inductor media inhibited the proliferation of induced differentiated cells **(C)**. Stem cell markers (CD90 and CD117) were also found to be lower after differentiation via flow cytometry **(D).** Adipogenic, chondrogenic, osteogenic and neurogenic differentiation of KATO-III was confirmed by coloration **(E).**

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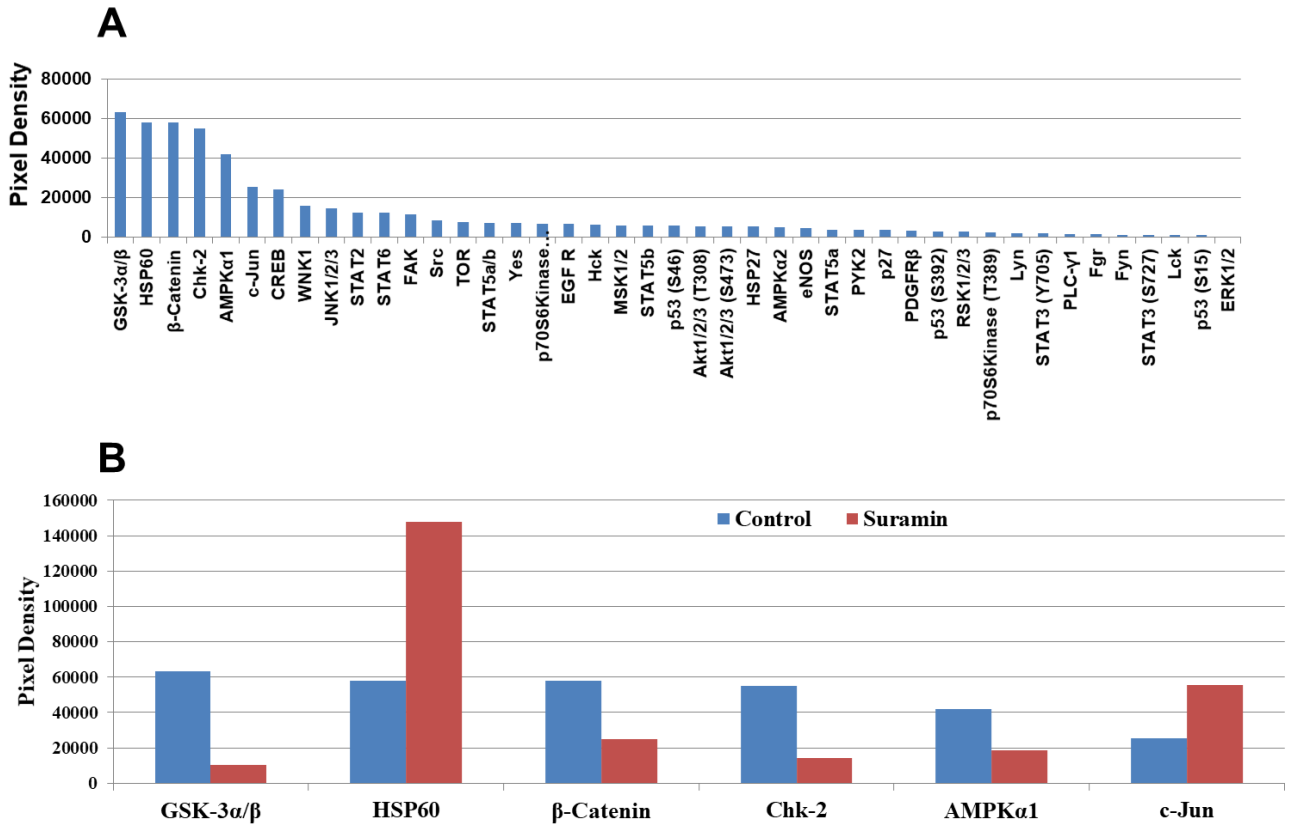
Suramin modified phosphokinase activity pattern of KATO-III cells



Phospho-kinase array in control and treated (Heparanase, suramin or both) KATO-III cell line.

6 different kinases were observed in KATO-III treated with suramin (200µM) in comparison to the control. After treatment, 2 were upregulated (HSP60 and C-Jun) and 4 (GSK-3α/β, β-catenin, Chk-2 and AMPkal-1) were down regulated.

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Conclusion

In the scenario of a progressive gastric fibrosis like signet ring cell adenocarcinoma of stomach, HPSE (which is increased in gastric microenvironment) is responsible for pro-fibrotic factor dependent EMT leading to cell malignancy and fibrosis. The expression level of LRP and MDR-1 was found higher in signet ring cell adenocarcinoma which contributes to the chemoresistance observed in this malignancy. Suramin has been shown to be effective in the prevention and treatment of the EMT related fibrosis. HPSE could therefore be an interesting pharmacological target for the treatment of gastric signet ring cell adenocarcinoma.