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In vitro evaluation of multi-targeted kinase inhibitors in scirrhous gastric cancer cell lines by tyrosine kinase activity profiling

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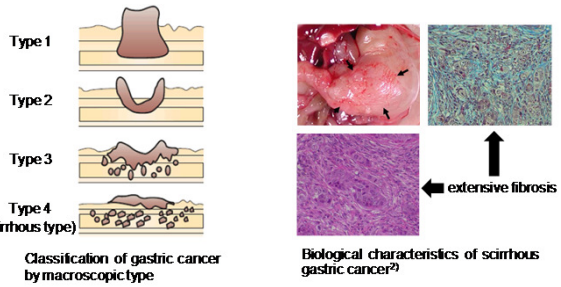
Introduction

Gastric cancer is one of the leading causes of cancer-related deaths in Japan and East Asia. Scirrhous-type gastric cancer, which accounts for about 10% of all gastric cancers, interacts with stromal cells with involvement of multiple growth factors. Common features of scirrhous-type gastric cancer include rapidly progressive invasion and a high frequency of metastasis to the peritoneum. Although a fluoropyrimidine and platinum-containing regimen has been established as the standard chemotherapy, its efficacy is not satisfactory. Combined chemotherapy with multi-targeted kinase inhibitors (MTKIs) such as sunitinib has been recently reported to be effective in a subset of patients with scirrhous-type gastric cancer but no predictive biomarker or companion diagnostics has been developed[1)].

Here we examined the efficacy of the MTKIs in scirrhous gastric cancer cell lines and conducted an exploratory predictive biomarker study by tyrosine kinase activity profiling with PamChip® peptide micro-arrays.



Classification of gastric cancer by macroscopic type

Biological characteristics of scirrhous gastric cancer[2)]

Materials and Methods

- 11 scirrhous & 14 non-scirrhous gastric cancer cell lines were used (Table 1).
- The growth-inhibitory effect of the MTKIs sunitinib, sorafenib and pazopanib was evaluated by the MTT assay (Table 2 and Fig. 3).
- Expression levels of target molecules of MTKIs in cell lines were evaluated by immunoblot analysis (Fig. 4).
- Cells were grown until semi-confluent, lysed, aliquoted and stored at -80˚ C until use. The tyrosine kinase activity of the lysates was measured in the absence and presence of the MTKIs on PamChip® peptide micro-arrays, containing 144 peptides derived from known human phosphorylation sites using 10 µg of total protein per test.
- Data analysis and statistical analysis were carried out with BioNavigator software according to the inhibition profiles calculated for each MTKI by taking the log-ratio of peptide phosphorylation measured in the absence and presence of the MTKIs.

Table 1. Gastric cancer cell lines

Name	Scirrhous/Non-scirrhous	Histology
HSC-39	Scirrhous	Signet-ring cell carcinoma
HSC-40A	Scirrhous	Signet-ring cell carcinoma
HSC-43	Scirrhous	Signet-ring cell carcinoma
HSC-44PE	Scirrhous	Signet-ring cell carcinoma
HSC-45	Scirrhous	Signet-ring cell carcinoma
HSC-58	Scirrhous	Signet-ring cell carcinoma
HSC-59	Scirrhous	Signet-ring cell carcinoma
HSC-60	Scirrhous	Signet-ring cell carcinoma
44As3	Scirrhous	Signet-ring cell carcinoma
58As1	Scirrhous	Signet-ring cell carcinoma
KATOIII	Scirrhous	Signet-ring cell carcinoma
OCUM-1	Non-scirrhous	Signet-ring cell carcinoma
JR-st	Non-scirrhous	Signet-ring cell carcinoma
NUGC-3	Non-scirrhous	Poorly + signet ring component
NUGC-4	Non-scirrhous	Poorly + signet ring component
SNU-1	Non-scirrhous	Poorly-differentiated adnocarcinoma
SNU-16	Non-scirrhous	Poorly-differentiated adnocarcinoma
MKN45	Non-scirrhous	Poorly-differentiated adnocarcinoma
OKAJIMA	Non-scirrhous	Poorly-differentiated adnocarcinoma
IM95	Non-scirrhous	Moderately-differentiated adnocarcinoma
MKN74	Non-scirrhous	Moderately-differentiated adnocarcinoma
MKN28	Non-scirrhous	Moderately-differentiated adnocarcinoma
NCI-N87	Non-scirrhous	Well-differentiated adnocarcinoma
MKN7	Non-scirrhous	Well-differentiated adnocarcinoma
MKN1	Non-scirrhous	Adenosquamous cell carcinoma

Table 2. Multi-targeted kinase inhibitors and target molecules

Agent	Target	Phase of development
Sunitinib	VEGFR-1, -2, -3, PDGFR, KIT, FLT3, CSF-1R, RET	Approved for kidney cancer and GIST
Sorafenib	VEGFR-2, -3, PDGFR, KIT, Raf	Approved for kidney cancer and liver cancer
Pazopanib	VEGFR-1, 2, 3, PDGFR, KIT	Approved for kidney cancer

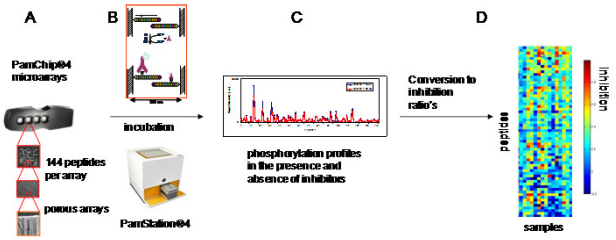


Fig. 1. Dynamic peptide micro-array technology. (A) PamChip®4 peptide microarrays. (B) The phosphorylation reaction takes place on peptides spotted on the array. Peptide phosphorylation through active kinases or inhibition thereof can be monitored in lysates of cell lines by real time fluorescent readout using a fluorescent labeled phospho-tyrosine antibody[3-4)]. (C) Signal intensities per spot represented in a phosphorylation profile. (D) Phosphorylation profiles for multiple samples represented as a heatmap.

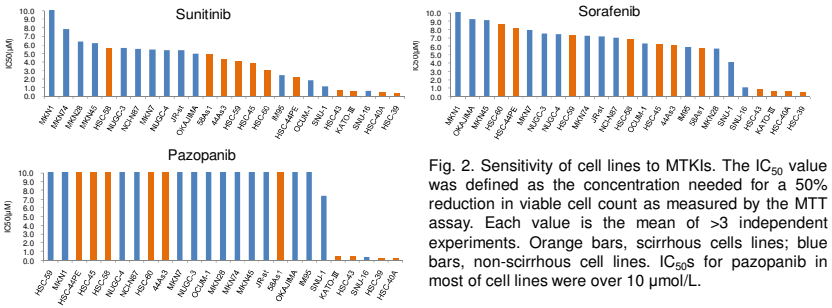


Fig. 2. Sensitivity of cell lines to MTKIs. The IC_{50} value was defined as the concentration needed for a 50% reduction in viable cell count as measured by the MTT assay. Each value is the mean of >3 independent experiments. Orange bars, scirrhous cells lines; blue bars, non-scirrhous cell lines. IC_{50}s for pazopanib in most of cell lines were over 10 µmol/L.

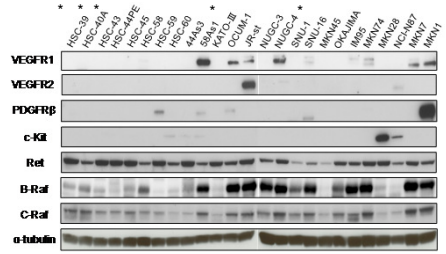


Fig. 3. Immunoblot analysis of expression levels of target molecules of MTKIs such as VEGFR1, VEGFR2, PDGFRβ, c-Kit, Ret, B-Raf, C-Raf and α-tubulin as an internal control in gastric cancer cell lines. The asterisks denote sensitive cell lines to MTKIs with IC_{50} value below 1 µmol/L.

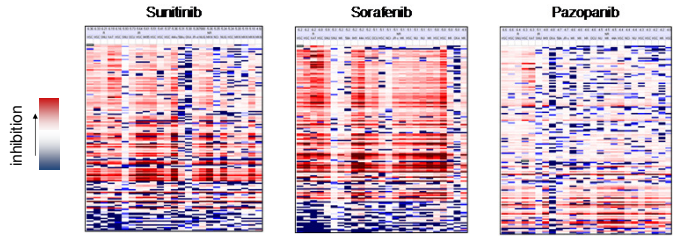


Fig. 4A. Color map representation of inhibition data. Cell lines are sorted according to their IC_{50} in the MTT assay and peptides according to correlation with IC_{50}s. For all 3 inhibitors a large number of peptides was found with $p < 0.01$ in a two sample t-test between responder and non-responder cell lines.

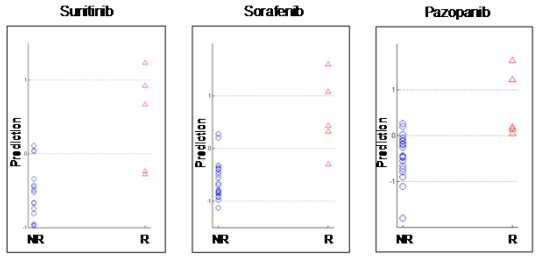


Fig. 4B. Partial least squares-discriminant analysis (PLS-DA) was used to correlate the *in vitro* inhibition on the PamChip® microarrays to the sensitivity of 25 gastric cancer cell lines to the MTKIs. Cell lines are classified as responders when the predicted value >0 and as non-responders when the prediction value < 0. Good predictive performance with a low misclassification rate was obtained with leave-one-out-cross-validation for all 3 inhibitors (MCR = 25%, 12%, 12.5%, respectively). NR, non-responder; R, responder.

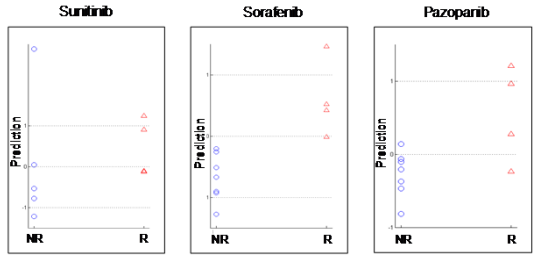


Fig. 4C. PLS-DA was used to correlate the *in vitro* inhibition on the PamChip® microarrays to the sensitivity of 11 scirrhous-type gastric cancer cell lines to the MTKIs. Cell lines are classified as in Fig. 4B. Good predictive performance with a low misclassification rate was obtained with leave-one-out-cross-validation for all 3 inhibitors. NR, non-responder; R, responder.

Conclusions

- MTKIs are efficacious in a subset of scirrhous-type gastric cancer cell lines. Sunitinib showed the most potent growth-inhibitory effect, suggesting that it should be considered for further evaluation in scirrhous-type gastric cancer.
- Phosphorylation profiles can be generated by measuring PTK activity in scirrhous-type and non-scirrhous-type gastric cancer cell lines using PamChip® peptide micro-arrays.
- Sensitivity to MTKIs in the MTT assay could be correlated to the *in vitro* inhibition of kinase activity on the PamChip® peptide micro-array.
- These data suggest that an *in vitro* assay on PamChip® peptide micro-array can serve as a companion diagnostic test for MTKIs in patients with scirrhous-type gastric cancer using clinical tumor specimens.
- Target molecules of MTKIs most of which are angiogenesis-related molecules does not seem to be a growth driver of gastric cancer cells including scirrhous type, suggesting their expression does not predict clinical efficacy.

References

1. Muro K et al., ESMO 2010
2. Yashiro M et al., Cancer Microenvironment 2010
3. Sikkema AH et al., Cancer Research 2009
4. Versele M et al., Molecular Cancer Therapeutics 2009