

# Comparative Image Analysis of Vimentin Immunohistochemical Expression in Sunitinib-Responsive and Sunitinib-Resistant Renal Cell Carcinoma Xenografts

Timothy W. Affolter, Scott Garza, and Beatrice DeBrosse-Serra

Drug Safety Research and Development,  
Pfizer-La Jolla, San Diego, California, 92121  
Email: timothy.affolter@pfizer.com

**Abstract**— Some cancer patients treated with sunitinib, a leading standard of care for renal cell carcinoma, eventually acquire some degree of resistance to treatment. This study was designed to investigate the possibility that acquired resistance may be due in part to epithelial to mesenchymal transition (EMT) of tumor cells. This study used mouse xenografts derived from internally developed sunitinib-resistant and sunitinib-sensitive varieties of 786-O renal cell carcinoma lines. Groups of mice with xenografts derived from sunitinib resistant or sensitive cell lines were either treated with sunitinib (60 mg/kg/day) or not, and necropsied after 60 days. Immunohistochemistry (IHC) to quantify vimentin levels in these cells, an indicator of mesenchymal differentiation, was performed and evaluated in different regions of the xenograft using multispectral automated image analysis technology (Cambridge Research and Instrumentation, Cambridge, MA). Tumor cells in the transitional regions of the xenografts where tumor interfaced with host tissue showed increased expression of vimentin when these cells were derived from sunitinib-resistant variants, both in comparison to the sunitinib-sensitive variants as well as in comparison to the remaining regions of the xenograft tumor in the same individual slides. In this xenograft model, increased vimentin expression in transitional regions and invasive fronts adjacent to host tissue renal cell carcinoma cells supports the possibility of resistance in association with epithelial-mesenchymal transition.

## I. INTRODUCTION

Sunitinib is a small molecule anti-angiogenic tyrosine kinase inhibitor currently used as a leading standard of care for renal cell carcinoma and certain gastrointestinal stromal tumors. A certain percentage of patients with aggressive varieties of both of these tumor types have developed varying degrees of resistance to the compound.

Recent investigative efforts have succeeded in developing acquired resistant cell lines derived from the 786-O renal cell carcinoma line which typically is sunitinib responsive experimentally. The immediate preceding investigative study looked at the effects of sunitinib treatment on mouse xenografts derived from either sunitinib

resistant or responsive 786-O lines, including histopathology and a panel of IHC expression markers.

The most revealing finding from the previous study was the difference in cell morphology and invasiveness in the regions of xenograft/host tissue transition or the leading edges of xenograft tissue. In the resistant groups irrespective of treatment, there was a more irregular transition region with more frequent independent islands of tumor cells surrounded by host tissue, more frequent extension and arborization of narrow cords of tumor cells into the host tissue, more frequent bizarre mitotic figures near the host tissue interface, and more cells with mesenchymal-like cytoplasmic shape, definition, and association with one another.

These observations suggested that mesenchymal transition or associated gene expression alterations could be a possible cause of resistance worth investigating, particularly since the activation of certain pathways associated with mesenchymal transition are known to confer increased tolerance of tumor cells to hypoxic conditions such as those promoted by anti-angiogenic effects[1].

## II. MATERIALS AND METHODS

### A. Animals and Basic Design

This study retained the four experimental groups from a previous internal study 09LJ048a. Each of these groups was comprised of nine female scid-bg mice, with two groups containing xenografts derived from 786-O derived sunitinib resistant cells implanted subcutaneously. one of these two groups was treated with sunitinib (60 mg/kg/day) and the other group was given vehicle control (0.2 cc/day PBS buffer). The two additional groups containing xenografts from 786-O derived sunitinib sensitive cells were also separated into those treated with sunitinib (60 mg/kg/day) or vehicle (0.2 cc/day PBS buffer). On

day 60, all animals were necropsied, xenografts were bisected, collected, and retained in 10% formalin.

### B. Immunohistochemistry

Immunohistochemistry was performed using a mouse monoclonal anti-vimentin antibody (Dako corporation, Carpinteria, CA) and required internal optimization. Slides were prepared from formalin retained xenografts from necropsy as described above and sectioned to include all regions of the xenograft and small amounts of adjacent host connective tissue.

### C. Multispectral Automated Image Analysis

Automated digital image analysis was performed using multispectral image capture via the Nuance camera (Cambridge Research and Instrumentation, Cambridge, MA), and image analysis technologies known as Vectra and Inform (Cambridge Research and Instrumentation, Cambridge, MA). This involved RGB and multispectral image capture of sections from all slides in the study, classification of tissue versus non tissue, and subsequent classification of xenografts into areas of necrosis, transitional/host tissue interface zone, remainder of viable tumor, host tissue, and other (non-tissue). This classification was under the direction of and quality monitored by a board-certified pathologist. The following step of automated analysis involved segmentation of nuclei and cytoplasm for the transitional and central zones of xenograft tumors, and quantitative assessment of DAB intensity in terms of optical density score in tumor cell cytoplasm in these regions, and data comparison of intensity differences between the four experimental groups.

## III. RESULTS

The resistant groups irrespective of sunitinib treatment showed a much darker average cytoplasmic vimentin DAB optical density/intensity in the transitional regions of the xenograft adjacent to host tissue as compared to all other regions of viable tumor (see table I). This regional difference was not identified in the two sunitinib responsive groups, regardless of vehicle or sunitinib treatment. The transitional regions were also much lower in average intensity in the responsive groups when compared to the resistant groups. The transitional areas were not always at the edge of the tissue to non-tissue on the section, as variable cuts allowed for bands of host tissue occasionally to course through central portion of the actual section, and the same intensity pattern was still identified regardless of orientation of the transitional zone in relation to the edge of all tissue on the slide.

TABLE I  
AVERAGE CYTOPLASMIC DAB OPTICAL DENSITY

| group                                          | DAB-OD<br>(x1000)<br>Transitional<br>zone | DAB-OD<br>(x1000)<br>Tumor Body |
|------------------------------------------------|-------------------------------------------|---------------------------------|
| Sunitinib-sensitive,<br>PBS vehicle            | 41.3                                      | 34.1                            |
| Sunitinib-sensitive<br>60 mg/kg/d<br>sunitinib | 43.1                                      | 38.9                            |
| Sunitinib-resistant<br>PBS vehicle             | 80.5                                      | 33.2                            |
| Sunitinib-resistant<br>60 mg/kg/d<br>sunitinib | 88.7                                      | 34.8                            |

## IV. DISCUSSION

Low levels of cytoplasmic vimentin expression was identified throughout a large majority of tumor cells in all groups, suggesting that for the 786-O line there is some degree of constitutive vimentin expression. Vimentin expression in 786-O lines is more aptly viewed as a variable continuum and not as an absolute [1]. Although vimentin is often considered a mesenchymal marker, very low constitutive expression of vimentin can be seen in many epithelial cell types, but frequently is increased in neoplastic ones. More marked increases are generally indicative of epithelial-mesenchymal transition or similar gene expression alterations in the cell. The average intensity for most tumor cells in this study was generally low-medium, but was very high in the transitional zones adjacent to and invading into host tissue in many of the sunitinib-resistant derived xenografts. This supports the possibility of an epithelial to mesenchymal transition, or a similar gene expression pattern, and this type of transition could involve some genes that are conferring hypoxic resistance and/or increased mobility to the tumor cells, thus rendering them less susceptible to the anti-angiogenic and/or other effects of sunitinib treatment. A recent study evaluating gene expression data from a similar internal study also highlights increased expression of genes associated with mesenchymal transition. These findings could have important implications for directing the choice of co-therapies designed to combat the development of and/or survival of sunitinib resistant cells during treatment.

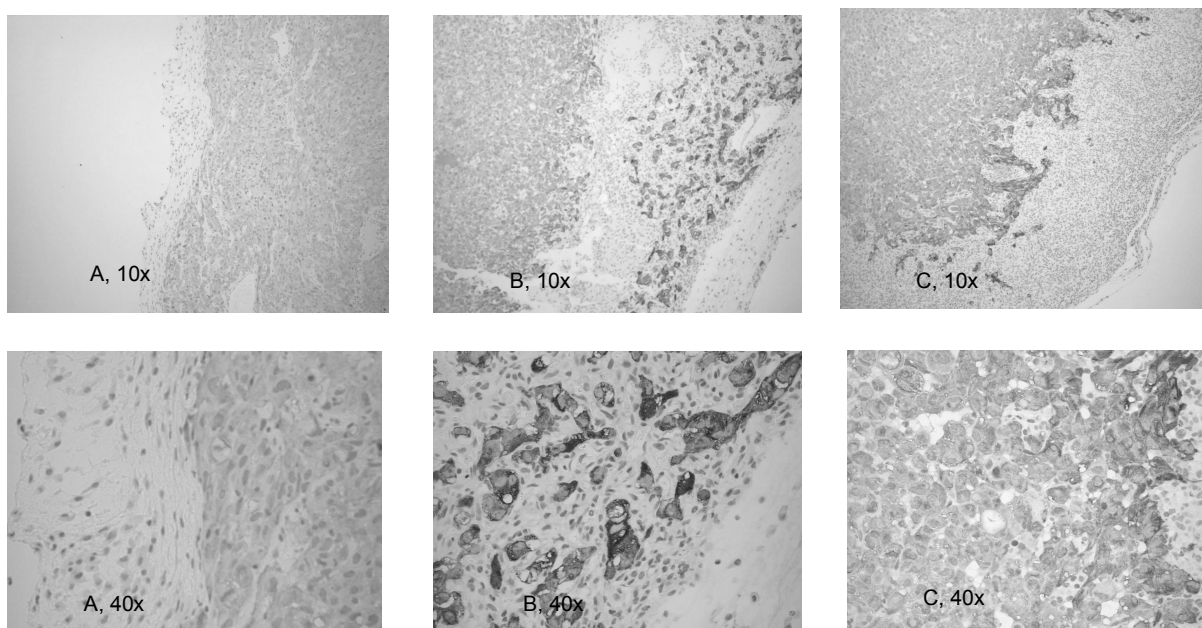


Fig.1. Differences in transitional zone morphology and differences in vimentin IHC expression between sunitinib-resistant and sunitinib-responsive xenografts. A= sunitinib responsive (non-treated) B=sunitinib resistant (non-treated) C=sunitinib resistant (treated). Note the relatively uniform staining throughout the tumor cells in A, whereas in B and C there is a darker staining pattern in tumor cells closer to or enveloped by host mouse tissue (the paler regions at the left edge of A, or to the right side of images B and C).

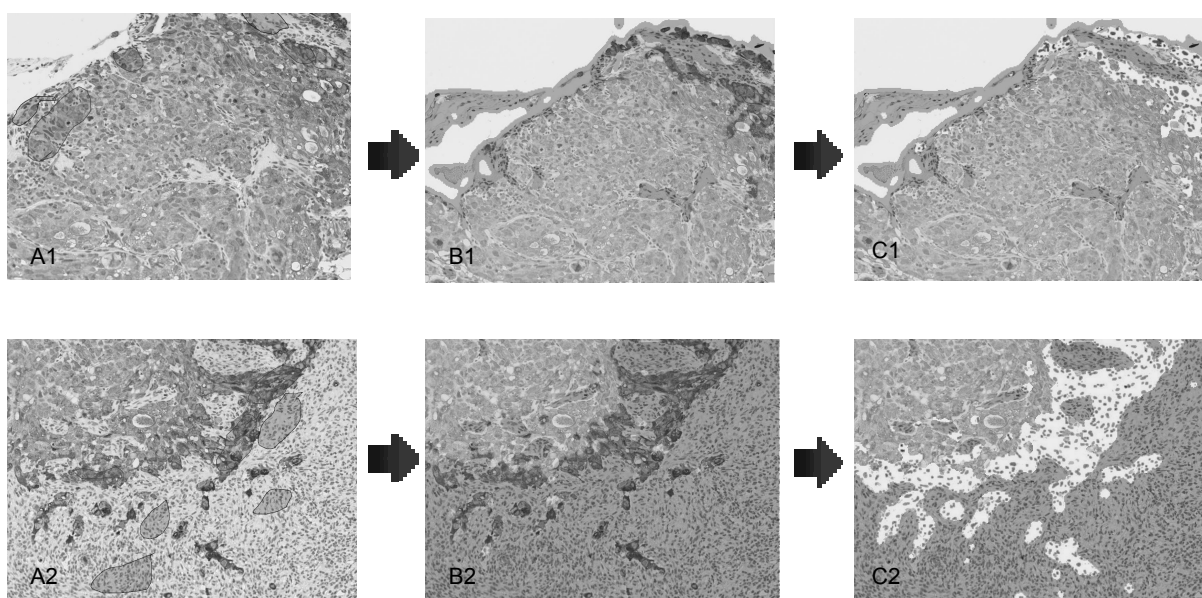


Fig.2. Multispectral automated image analysis of vimentin immunoreactivity in transitional zones. A=classification of subregions of the xenograft tumor during the training process (note different darker circles in A1 showing user definition of transitional regions and host tissue, and the lighter circles in A2 showing user definition of tumor body). B=automated software classification on another image following training, with different colors assigned to non-tissue, mouse tissue, tumor body, and transitional zone. C=shows segmentation of nuclei and cytoplasm in the lighter colored area in the center corresponding to a transitional region.

## V. CONCLUSION

The increased vimentin immunoreactivity of tumor cells in the host-tissue interface transitional regions when compared to the remainder of the xenografts in the sunitinib resistant cell lines suggests an epithelial-mesenchymal or similar transition of these cells, which may be responsible in part for conferring resistant properties to these cells. These cells also tend to arrange in more invasive patterns, or as small isolated islands or individual cells, suggesting increased invasive and/or metastatic potential. Particularly as gene expression data from another recent study also appears to support this finding, this may have important implications for the development of co-therapies designed to combat resistance. The image analysis software proved to be a successful and highly useful tool in the attainment and quantification of these conclusions.

## ACKNOWLEDGMENT

The authors would like to thank Anthony Wong and Dusko Trajkovic for assistance with immunohistochemistry optimization. Additional thanks to Cliff Hoyt and Craig Lassy at Cambridge Research and Instrumentation for assistance with software training and data interpretation.

## REFERENCES

- [1] Chuang MJ, Sun, KH, Tang SJ, Deng MW, Wu YH, Sung JS, Cha TL, and Sun GH. Tumor-derived tumor necrosis factor- $\alpha$  promoted progression and epithelial-mesenchymal transition in renal cell carcinoma cells. *Cancer Sci.* 2008 99(5): 905-13.
- [2] Guo T, Hajdu M, Agaram NP, Shinoda H, Veach D, Clarkson BD, Maki RG, Singer S, Dematteo RP, Besmer P, Antonescu CR. Mechanisms of sunitinib resistance in gastrointestinal stromal tumors harboring KITAY502-3ins mutation: an in vitro mutagenesis screen for drug resistance. *Clin Cancer Res* 2009 15(22): 6862-70.