Dear Editor Norton:

We are pleased to submit for your review our revised manuscript, “PI3K Activation is Associated with Intracellular Sodium/Iodide Symporter Protein Expression in Breast Cancer.” We greatly appreciate the comments and suggestions provided by reviewers Dr. Kogai and Dr. Bidart and have incorporated requested changes into our manuscript.

Reviewer #1: Dr. Bidart commented favorably on the extension of our earlier work on sodium/iodide symporter (NIS) regulation by PI3K and cAMP in transgenic mouse models into the MCF-7 cell system and human breast cancer tissue microarray. Specific comments are addressed as follows:

- **Comment #1:** “The clinical interest of these observations is less evident when comparing to new molecular targeted therapeutic and imaging strategies.”
  
  We agree that many exciting and novel methods for the treatment of breast cancer are in development. Our goal is that NIS-mediated radionuclide ablation will be among these methods. Further investigation into the mechanism(s) of impairment of NIS cell surface trafficking by PI3K will be critical in realizing the application of NIS-mediated radioiodide imaging and ablation in breast cancer.

- **Comment #2:** “The exact number of tissues analyzed for NIS interpretation should be precised.”
  
  While our array consisted of 36 tumors for NIS evaluation, only 33 had corresponding pAkt data. We have clarified this point in our revised manuscript.

- **Comment #3:** “The sentence ‘there was a 3-fold increased in Akt phosphorylation is somewhat misleading’...It is also difficult to conclude on the subcellular localization of NIS due to the low number of samples (n=2 for membranous NIS).”
  
  We have corrected this statement to clarify that NIS-positive tumors were three times more likely than NIS-negative tumors to have PI3K activation as indicated by Akt phosphorylation. We have also modified our statements regarding surface versus intracellular NIS and have sorted the NIS-positive tumors into only two categories, “intracellular (only)” and “plasma membrane (any).”

Reviewer #2: Dr. Kogai felt that the post-translational effect of PI3K on NIS in breast cancer was a novel finding. Specific comments are addressed as follows:

- **Comment #1:** While Figure 1B (cell surface biotinylation NIS Western blot) shows a lack of NIS on the cell surface of PI3K-activated MCF-7 cells, Figure 1C (NIS immunofluorescence) appears to show cell surface NIS in these cells. The morphology is also different from tRAH-treated parental MCF-7 cells.
  
  Indeed, the morphology of MCF-7/PI3K stable transfectants is different from the tRAH-treated MCF-7 cells, and the photos shown are representative of the cell populations. However, we have never found intense cell border NIS staining in the MCF-7/PI3K stable transfectants as shown in the tRAH-treated MCF-7 cells. We have modified our statement in the revised manuscript accordingly.

- **Comment #2:** NIS in breast cancer has been reported to be a 70 kDa – 100kDa protein in human and rodent models, while PI3K-activation generates a 50kDa NIS form in MCF-7 cells. Please discuss the discrepancy.
  
  This is an interesting point. NIS glycosylation status appears to be quite different between human and mouse. For example, NIS in four human breast tumors was shown to be 97kDa with intracellular NIS localization (Upadhyay et al., 2003), yet NIS in the mouse mammary gland and
mouse mammary tumors was shown to be around 70kDa (Tazebay et al. (2000) with evident cell surface localization (Knostman et al. 2004). For mammary gland tumors in vivo, where multiple genetic mutations and hormones are involved, the signaling cascades are likely much more complicated than our cell model.

The fact that MCF/PI3K stable transfectants express only 50kDa NIS may be due to its highly manipulated condition. Most importantly, MCF-7 cells transiently transfected with activated PI3K also results in an increase in underglycosylated 50kDa NIS. It is unknown whether underglycosylated NIS was also present in the four human breast tumors in the paper published by Upadhyay et al., as it did not include the lower molecular weight portion of the Western blot. In addition, PI3K status of these human tumors was not reported.

- **Comment #3:** Cells stably transfected with empty vector should be compared with cells stably expressing PI3K to rule out artifact caused by G418 selection and insertion of exogenous DNA.
  While we do not currently have MCF-7 cells stably expressing pcDNA3, we do not believe that our finding is an artifact of G418 selection. Most convincingly, we showed that transient transfection of activated PI3K leads to an increase in underglycosylated NIS (Fig 2A).

- **Comment #4:** Up to 50% decreased NIS function has been reported after mutagenesis of NIS glycosylation sites. Are there any other mechanisms to reduce NIS trafficking with PI3K activation? The contribution of reduced NIS trafficking and function due to deglycosylation should be discussed.
  We interpret the lack of NIS glycosylation in our MCF-7 model to be a consequence of protein retention at a pre-glycosylation stage rather than a cause of trafficking failure. However, we cannot exclude that a lack of glycosylation itself might impair trafficking and function. We now address this possibility in our discussion section.

- **Comment #5:** Since a correlation between nuclear pAkt and intracellular NIS is suggested in immunohistochemistry, representative staining of the pAkt and NIS should be shown.
  We apologize for our previous confusing statement. In response to Dr. Bidart’s comment #3 above, we have sorted NIS-positive tumors into only two categories. Accordingly, we do not find a statistically significant correlation between nuclear pAkt and restricted intracellular localization of NIS.

- **Comment #6:** The method for densitometry in Figure 2 should be described.
  We have added to our methods section the use of Scion scanning for densitometry.

We hope that our revised manuscript now meets the criteria for publication in BMC Cancer. Please contact us with any questions.

Sincerely,