Final Progress Report ANN 110922

Title: Ca^{2+} signaling in BRAF mutant tumors: therapy resistance and drug combinations

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Abstract

Mutations in the BRAF gene in human cancer are often associated with an aggressive phenotype, high metastatic potential and poor prognosis. Although BRAF inhibitors have been proven to be highly effective in melanoma treatments, such inhibitors were particularly ineffective in colorectal cancer (CRC) and other solid tumors. Even in melanoma there remains a large group of patients who do not respond to BRAF inhibition therapies or develop resistance quickly, requiring further exploration for new treatment options. With the support of the past research grant our group in collaboration with the laboratories at the Medical University of Vienna was the first who identified the plasma membrane Ca^{2+} ATPase PMCA4b as a putative metastasis suppressor in BRAF mutant melanoma cells. We found that inhibition of mutant BRAF increased the expression of this Ca^{2+} pump, which then inhibited the migratory and metastatic potential of the cells. We further showed that in melanoma cells the expression of PMCA4b was under epigenetic control and treatment with HDAC inhibitors led to elevation of PMCA4b expression. The increased PMCA4b level was coupled with inhibition of the migration of BRAF mutant melanoma cells to an extent comparable to that seen after treatment with the mutant BRAF inhibitors. Our results shed light on a previously unidentified role of the PMCA4b protein in tumor progression that may provide deeper understanding of the metastatic process together with additional prognostic and therapeutic options for these deadly BRAF mutant cancers.

Introduction and specific aims

The calcium ion is an important second messenger within the living cell, where it regulates such opposing processes as cell proliferation and cell death. The messenger function of Ca^{2+} is made possible by the existence of a steep concentration gradient between the cytosol and the intracellular and extracellular compartments that is maintained by active Ca^{2+} transporters or pumps. Among these pumps the plasma membrane Ca^{2+}ATPase (PMCA, encoded by the ATP2B gene family), a widely known gatekeeper at the plasma membrane, is responsible for removing the excess Ca^{2+} from the cytosol, hence preventing cells from calcium overload.

Cellular Ca^{2+} homeostasis is often remodeled during tumorigenesis that can result in an imbalance of survival and cell death. It has been suggested that the *ATP2B4* gene (encoding PMCA4) plays a key role in this process (see our review in ref 4: *Padányi et al., BBAmcr, 2016*). The support of the past research grant enabled us to

analyze the expression and specific role of the PMCAs in BRAF mutant melanoma cells, and extend these studies to other BRAF mutant tumors such as colorectal tumors. Our aims have been to *1./ explore the mechanism by which mutant BRAF specific inhibition altered Ca^{2+} signaling in BRAF mutant cells; 2./ determine how changes in Ca^{2+} signaling affect downstream signaling pathways of BRAF mutant tumor cells; 3./ study how Ca^{2+} level modulation and BRAF inhibition affect migration, proliferation and tumor growth; 4./ establish the pathological relevance of PMCA protein expression in BRAF mutant cancers.*

Collaboration

With the help of the past research grant support a very successful international collaboration among Dr. Enikő Kállay, Institute of Pathophysiology and Allergy Research, Dr. Michael Grusch, Cancer Research Institute of the Medical University of Vienna (MedUni Wien), and Dr. Agnes Enyedi, Tumor Progression Research Group, 2nd Institute of Pathology, Semmelweis University (SE), Budapest has been developed. The successful collaboration has led to the novel important finding that PMCA4b is a putative metastasis suppressor.

Major new findings supported by ANN 110922

1. PMCA4b is selectively up-regulated in BRAF mutant melanoma cells after BRAF and MEK inhibition. In these experiments we used three BRAF wild type (MEWO, HOST, MJZJ) and two BRAF mutant (A375, A2058) cell lines and studied PMCA expression both at the protein and mRNA levels in response to BRAF (vemurafenib and dabrafenib) and MEK inhibitor treatments. These treatments induced a substantial increase in PMCA4b expression both at the mRNA and protein levels in the BRAF mutant cells while no change in the PMCA4b expression level was observed in the BRAF wild type cells under similar conditions. These data revealed a crosstalk between Ca^{2+} signaling and the MAPK pathway through the PMCA.

We also analyzed the expression of PMCA proteins in colon cancer cells using two BRAF mutant (HT29, WiDr), two KRAS (DLD1, SW480) mutant and two BRAF and KRAS wild type cell lines (Caco2, HCA7). We found that MEK inhibitor treatment increased – although slightly – PMCA4b expression in all BRAF and KRAS mutant cell lines whereas vemurafenib treatment affected PMCA4b expression only in the BRAF mutant WiDr cells. The lesser response of these cells to these inhibitor treatments is in good accordance with previous observations that BRAF mutant colorectal cancer (CRC) cells are often resistant to BRAF inhibitors. The results regarding the regulation of PMCA4B expression in CRC cells will form the basis of a further publication.

The effect of MAPK inhibitors on PMCA4b expression was specific as vemurafenib treatment did not affect significantly the expression of other elements of the Ca^{2+} signaling toolkit in these cells. We found that the mRNA expression levels of neither the inositol 1,4,5-triphosphate receptor type 1-3 (IP3R1, IP3R2, IP3R3), ORAI calcium release-activated calcium modulator 1 (ORAI1), ryanodine receptor 2 (RYR2), stromal interaction molecules 1 and 2 (STIM1, STIM2) nor the transient receptor potential cation channel subfamily

M member 1 (TRPM1) was altered under these conditions. These results underline the importance of PMCA4b upregulation in response to inhibitors of the MAPK pathway in the BRAF mutant cell lines.

2. **Increased plasma membrane expression of PMCA4b was associated with an enhanced Ca^{2+} clearance.** Confocal microscopy analysis demonstrated that vemurafenib treatment substantially increased the level of PMCA4b in the plasma membrane of BRAF-mutant melanoma cells. To study how PMCA4b affected Ca^{2+} signaling store-operated Ca^{2+} entry (SOCE) was induced after store depletion by using the Ca^{2+} re-addition protocol (ref 8: *Pászty et al. Science Signaling, 2015*). In the vemurafenib-treated BRAF mutant cells, intracellular Ca^{2+} concentration declined to the basal level much faster after the SOCE peak than in the untreated cells, whereas vemurafenib had no effect on Ca^{2+} clearance in BRAF wild type cells.

3. **Vemurafenib treatment inhibited migration and proliferation of BRAF mutant melanoma cells.** We examined the effect of vemurafenib treatment on the migratory activity of a BRAF wild type and two different BRAF mutant melanoma cell lines. The migration of the cells was recorded by time-lapse video microscopy. We showed that vemurafenib slowed down substantially the migration of the BRAF mutant cells while it did not change that of the already slow BRAF wild type cells. We also demonstrated that vemurafenib treatment strongly decreased proliferation of the BRAF mutant cells while it did not affect that of the BRAF wild-type cells.

4. **PMCA4b over-expression decreased the migratory activity of BRAF mutant cells but did not affect their proliferation rate.** To study if PMCA4b was responsible for the decreased cell motility we generated BRAF mutant A375 cell lines stably expressing EGFP-tagged PMCA4b or EGFP alone. On the one hand, we found that the PMCA4b over-expressing BRAF mutant A375 cells had altered cell morphology and a marked decrease in their migratory activity as compared to the parental cells. On the other hand we demonstrated that PMCA4b over-expression did not affect cell proliferation. We did not find any difference in the mRNA expression of EMT marker proteins (E-cadherin, ZEB1, Snail and vimentin) between control EGFP and EGFP-PMCA4b over-expressing cells suggesting that these proteins were not involved in the cell morphology and motility change.

5. **NFAT and MITF were not involved in the phenotypic changes of BRAF mutant melanoma cells.** As NFAT is a downstream target of Ca^{2+} signaling we analyzed the mRNA expression levels of the NFAT 1-4 genes in BRAF mutant cells but only NFAT4 was detectable and its expression did not change after vemurafenib treatment or PMCA4b overexpression. The microphtalmia-associated transcription factor (MITF) is a known regulator of melanocyte development but no significant change in its mRNA expression was detected either. These data indicated that neither NFAT nor MITF were involved in the observed alterations in morphology and migratory activity of these BRAF mutant cells.

6. **PMCA4b expressing BRAF mutant cells had significantly lower metastatic potential *in vivo*.** The reduced migratory characteristic of the PMCA4b expressing BRAF mutant cells was associated with a marked decrease in their metastatic activity *in vivo*, as demonstrated in a mouse lung colonization assay. Altogether, these data suggest that PMCA4b is a putative metastasis suppressor of BRAF mutant melanomas.

7. **Gene expression microarray data showed decreased PMCA4b expression in cutaneous melanoma compared to benign nevi.** In order to demonstrate that PMCA4 expression is related to the malignant progression of melanoma, we analyzed two datasets in the ONCOMINE database where benign nevi and melanoma specimens could be directly compared. In both datasets the proportion of cases with high PMCA4 expression was higher in benign nevi than in melanoma cases. Pooling the data from the two cohorts, 21 out of 27 (77%) benign nevi had high PMCA4 expression in contrast to 26 out of 69 (38%) melanomas. The results of 1-7 are published in ref 3: *Hegedűs et al. IJC, 2017.*

8. **Histone deacetylase inhibitors (HDACis) increased the expression of PMCA4b in melanoma cells independent of their BRAF status.** Recent experiments suggested that misregulated HDAC activity can affect PMCA4b expression therefore we treated BRAF mutant and BRAF wild type melanoma cells with the HDAC inhibitors, valproate and SAHA (suberoylanilide hydroxamic acid). We found that treatment with the HDAC inhibitors increased the level of PMCA4b expression at both the mRNA and protein levels in both BRAF wild type and BRAF mutant cell types. The increased PMCA4b level was coupled with an enhanced plasma membrane localization, Ca^{2+} clearance and reduced cell motility without having a substantial influence on cell proliferation. Specific inhibition of PMCA4b activity partially blocked the effect of HDACis underlining the role of PMCA4b in the regulation of cytosolic Ca^{2+} concentration and cell motility. Our results show that in melanoma cells the expression of PMCA4b is under epigenetic control and HDAC inhibitors efficiently enhance PMCA4b expression independent of the BRAF status of cells. These results are published in ref 1: *Hegedűs et al. Frontiers in Oncology, 2017*.

9. **Reduced red blood cell PMCA4b level was associated with a minor haplotype in the PMCA4b gene.** In collaboration with the group of Balázs Sarkadi (MTA-TTK, Budapest) we found that about 10 % of healthy volunteers has significantly reduced expression of PMCA4b in their red blood cells. This low erythrocyte PMCA4b protein level was correlated with a formerly unrecognized minor haplotype in the predicted second promoter region of the ATP2B4 gene. Interestingly, SNPs in this *ATP2B4* haplotype have been linked to reduced mean corpuscular hemoglobin concentrations (MCHC), and to protection against malaria infection (the results are published in ref 2*: Zámbó et al., Cell Calcium, 2017*).

Impact

The collaboration between the two Institutions, SE and MedUni Wien, has been a great success and the benefits for both sides have been substantial. The collaboration yielded 2 joint papers in high impact journals, and 6 additional publications of the PI's laboratory were supported fully or partially by the present grant (cumulative IF: 39). Also, the joint project has resulted in 4 oral and 10 poster presentations at International and National conferences. By the end of 2017 four students will complete their master thesis in the Enyedi laboratory. Similarly, three master students have been supported by this grant in the laboratories of the MedUni Wien. In addition, Luca Hegedűs completed her PhD dissertation this year using the results of the past research grant support. She has received a travel award from the Comprehensive Cancer Center (MedUni

Wienna) to attend the Annual Meeting of the American Association for Cancer Research in New Orleans, 16-20 April 2016. In 2017 an MSc student from the Enyedi laboratory spent three months in the Grusch laboratory and in return two MSc students visited the Enyedi laboratory with the support of the past research grant and the Erasmus+ program of the European Union.

References:

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