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Characterization of the Anterior Gradient 2 homolog (AGR2) and the Anterior Gradient 3 homolog (AGR3) proteins in prostate cancer cell lines

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Prostate cancer (PCa) is the most common solid tumor and the third leading cause of male cancer deaths in developed countries. Because of the public health problem that this represents, screening tests for prostate cancer are under investigation. Prostate Specific Antigen (PSA) is the most widely used tumor marker in this regard. However, PSA testing detects both aggressive tumors as well as indolent cancers that might otherwise escape clinical diagnosis. Disadvantages of PSA such as low specificity have shown the necessity for the development of additional approaches for detection and prognostication.

The anterior gradient homologue 2 (AGR2 or HAG2) and the anterior gradient homologue 3 (AGR3 or HAG3) are two secreted proteins that share about 71% homology. Previous work in our group could identify that these proteins were up-regulated in cell extracts of prostate cancer in concordance with other scientists that profiled AGR2 as a novel tumor marker in various malignancies. AGR2 also has been proposed to play an important role in proliferation and invasion when tested *in vitro* and *in vivo* as well as for cell survival. In the case of AGR3 there is not a lot of scientific information available concerning its function and secretion.

Two principal applications have been proposed for these proteins, one, as tumor markers and second, as new targets for therapy. To study these two proteins and to contribute to the development of new tools for the treatment as well as early detection of PCa, we firstly developed a methodology to approach the high degree of homology between AGR2 and AGR3 and afterwards we studied the secretion of these proteins, expression induced by androgens, degradation, and their role in cell survival.

To start the current project, firstly we needed to correct the sequence of AGR3 that in our library was mutated by the substitution of C by A at position 473 in order to clone it into an E. coli expression plasmid for the recombinant production of AGR3. After this correction recombinant AGR2 and AGR3 were produced to test previously available antibodies in our lab. Thus, it was possible to recognize those antibodies which were specific for one of the two proteins or those that were unspecific. While an excellent AGR2 antibody was available, our experiments showed that no specific AGR3 antibody existed. Therefore, novel AGR3-antibodies were generated in rats using optimized peptide sequences. We then characterized AGR2 and AGR3 in prostate cancer cell lines PC3, DU145, LNCap, DuCap and 22Rv1 to understand the biology of these two proteins. Western Blot analysis showed that LNCap, DuCap and 22Rv1 cells expressed AGR3 and PC3, LNCap and DuCap were positive for AGR2. To demonstrate that our proteins of interest were secreted, several protein precipitation protocols were used. Thereby it was possible to document the presence of AGR2 and AGR3 in the supernatant of 22RV1 and DuCap cell lines. Since prostate cancer is highly androgen dependent, AGR2 and AGR3 expression was also tested in the presence and absence of dihydrotestosterone (DHT). Western blot from total cell lysate and supernatant of DuCap showed that AGR2 is up-regulated by androgens, whereas AGR3 is not strongly stimulated. This data was confirmed by real time PCR. Using 22Rv1 cell lines we were able to demonstrate that AGR3 is constantly produced and secreted. While AGR2 seems to be degraded by the proteasome pathway under the influence of DHT, this effect could not be documented for AGR3. To test the effects of a transient knockdown of AGR3 in 22Rv1 cells (cells that express AGR3 exclusively) we established a transfection methodology and then we examined proliferation, cell cycle and apoptosis induction. The results of this approach showed that the total count of 22Rv1 cells was reduced in those cells that where transfected with AGR3 siRNA in comparison with the respective control. To explore the reason for this reduction, we investigated two possible scenarios: cell cycle interruption and induction of apoptosis. Cell cycle analysis showed no difference between cells transfected with AGR3 siRNA and control siRNA. However, analysis of the surface presence of Annexin V showed an increased percentage of cells going into the apoptotic state after AGR3 knockdown meaning that the reduction of the total cell count is likely due to an increase in apoptosis in cells that are lacking AGR3.

In conclusion, AGR2 and AGR3 are currently being investigated for their possible role as tumor markers because of their secretion and overexpression in certain types of malignancies. In our work we demonstrate that care must be taken to assure specificity of antibodies used for detection of each protein to avoid cross reactivity. We were able to create antibodies that specifically detect AGR2 and AGR3. Using these we were able to show that AGR2 and AGR3, although being very similar proteins, are differently influenced by DHT. Knockdown of AGR3 in 22Rv1 cells reduced the cell number of viable cells but had no influence on cell cycle indicating that AGR3 may be necessary for cells to survive under stress conditions. Further experiments to elucidate the importance of these proteins will be performed in the future to clarify their roles and marker potential in many cancers and especially in PCa.