Influence of the C5a Receptor on Ovarian Cancer Progression

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Introduction:

The complement system is part of the innate immune system that is made up of distinct plasma proteins that react with each other to opsonize pathogens and cause an inflammatory response that helps fight infection (Janeway, et. al., 1996). C5aR is a G protein-coupled receptor that regulates inflammatory responses. It is a very potent inflammatory response molecule, which is beneficial in the process of clearing infectious disease because of the gathering of immune cells. Overproduction of C5aR, however, is known to be involved in the development of many inflammatory diseases such as IBS or RA (Imamura, et al., 2016).

In cancer, C5aR is known to be associated with tumor aggressiveness, as well as poor patient outcome, in cancers such as breast, endothelial, lung, and colon (Markiewski, 2014) (Imamura, et al., 2016) (Cho, et al., 2014). Cancer cells secrete complement proteins that stimulate tumor growth. When the complement system is activated inside of tumors, C5a attracts myeloid cells into the tumor, which reduces cytotoxic T cell responses to the tumor by promoting apoptosis and inhibiting CD8+ TILs, which are necessary for tumor elimination. The binding of C5a to C5aR on T cells has anti-apoptotic and pro-proliferative effects (Afshar-Kharghan, 2017).

The purpose of this study was to determine the influence of the C5a receptor in ovarian cancer progression. Ovarian cancer is the fifth leading cause of cancer death in the United States each year. It has been referred to as the silent killer, in that only 30% of women are diagnosed in the early stage. Early-stage ovarian cancer is confined to the ovary, and is more likely to be treated successfully (Mayo, 2019). Since 70% of women are diagnosed in the later stages of ovarian cancer it has a low survival rate (McCorkle, et al., 2003). Surgery and chemotherapy are used to treat ovarian cancer, but is more successful in early stages (Mayo, 2019). Learning about the effects that C5aR has on ovarian cancer is important, because aberrant expression of the C5a receptor has been such detected in cancers such as breast, lung, endothelial, and colon, but the influence of the C5a-C5aR axis on ovarian cancer progression has not yet been elucidated. If C5aR influences ovarian cancer progression, it can be targeted during ovarian cancer treatment.

Hypothesis: We hypothesize that C5aR protein expression level will increase with ovarian tumor grade.

Experimental Design:

A human paraffin embedded ovarian cancer tissue array with seventy-nine ovarian cancer cores, was stained immunohistochemically and given an Allred score. The slide contained five ovarian tumor types: serous papillary adenocarcinoma, mucinous adenocarcinoma, endometroid adenocarcinoma, clear cell carcinoma, and mucinous cystadenocarcinoma.

Immunohistochemistry: The slide was run through alcohols and then placed in a coplin jar full of Tris-EDTA (pH of 9.0). A steamer was used as the heat source for the heat-induced epitope retrieval. A pap pen was used to outline the tissue, then 3% hydrogen peroxide was placed on the tissue and the slide was washed in PBS. Blocking serum from ImmPRESS® Horse Anti-Rabbit IgG Polymer Kit (Vector) was incubated at RT for 45 minutes, then was incubated overnight at 4 degrees with rabbit anti-C5a-R (abcam) 1:100 in PBS. After incubation, the slide was washed in PBS, and then incubated again with secondary from ImmPRESS® Horse Anti-Rabbit IgG Polymer Kit for thirty minutes RT. The slide washed with PBS, and then incubated, with ImmPACT® NOVARED™ (Vector) for six minutes and thirty seconds RT. The slide was

then washed with water, counterstained with hematoxylin for forty-five seconds, washed again with water, and dehydrated. The slide was then cleared with xylene and mounted with cytoseal.

Allred score: The Allred scoring method is commonly used to score hormone receptor positive cancers, such as breast cancer. This scoring method involved determining the percentage of positive cells for the C5a receptor, as well as determining the intensity of the stain. This was done using light microscopy. The percentage (PS) was added to the intensity (IS) to obtain a score from 0 to 8 (Table 1) (EOD Data).

Table 1. Allred score definition

Proportion of positive staining score		Staining intensity score	
PS	Range (%)	IS	Type
0	0	0	No staining
1	<1	1+	Weak positive staining
2	1 – 10	2+	Moderate positive staining
3	11 – 33	3+	Strong positive staining
4	34 – 66	Allred score = PS + IS	
5	67 – 100		

Results:

Normal ovary tissue and normal lung tissue underwent immunohistochemical staining, with and without the primary anti-body, to act as controls. The lung was used as a control because endothelial cells line the pulmonary vasculature, and C5aR is normally found on some immune and endothelial cells. Since there is known expression of C5aR in these cells, they are a good positive control. The negative controls were not incubated with primary antibody, instead they were incubated with PBS. They underwent Nova red staining and a hematoxylin counterstain the same way the positive controls did. This allowed us to confirm that the staining was specific to C5aR. An Allred score was given to the ovarian controls.

Serous papillary adenocarcinoma (n=64) displayed good differential staining (figure 1 on poster). A bar graph summarizes the tumor grade vs. Allred score (figure 1.E). The Shapiro-Wilk test revealed that the data was normally distributed for tumor grade one (p=0.0580) but was not normally distributed for tumor grades two (p=0.0003) and three (p=0.0007). An ANOVA was done, since some of the data was normally distributed (p=0.2131). A Tukey Kramer test was done between the control and each tumor grade (1, 2, and 3), which revealed no statistical significance (1. P=0.9987, 2. p=0.0.8677, 3. p=0.8890). To further test significance, a linear regression test was done (figure 1.F) which revealed no statistical significance (p=0.0817).

Mucinous adenocarcinoma (n=10) displayed good differential staining (figure 2 on poster). A bar graph summarizes the tumor grade vs. Allred score (figure 2.E). The Shapiro-Wilk test revealed that the data was normally distributed for tumor grade two (p=0.5050), but the sample size was too small to test tumor grades one and three. The ANOVA revealed no significance (p=0.0990). A Tukey Kramer test was done between the control and each tumor grade (1, 2, and 3), which revealed no statistical significance (1. p=0.9565, 2. p=0.9960, 3. p=0.4206). To further test for significance, a linear regression test was done (figure 2.F) which revealed no statistical significance (p=0.1825).

Endometroid adenocarcinoma (n=3) displayed good differential staining (figure 3 on poster). A bar graph summarizes the tumor grade vs. Allred score (figure 3.D). The Shapiro-Wilk test was unable to be done because of the sample size being too small. The ANOVA revealed no significance (p=0.4264). A Tukey Kramer test was done between the control and each tumor grade (2 and 3), which revealed no statistical significance (2. p=0.8553, 3. p=0.4307). To further test significance, a linear regression test was done (figure 3.E) which revealed no significance (p=0.2391). In order to make a conclusion with this specific tumor type, the sample size would need to be increased drastically.

Conclusion:

The objective of this study was to stain a variety of ovarian cancer tumors for the C5aR, and analyze the amount of expression. It was hypothesized that the C5a receptor expression would increase with ovarian cancer tumor grade. There have been multiple studies in cancers such as breast, lung, endothelial, and colon cancer, that concluded that C5a-C5aR expression correlated with increased tumor grade and poor patient outcome (Markiewski, 2014) (Imamura, et al., 2016) (Cho, et al., 2014).

The immunohistochemical staining process in this study was successful. This is apparent because of the differential staining that was observed throughout the seventy-nine cores. We expected to see an increase of C5aR expression as tumor grade increased, but instead we saw no trend. There were several factors that could have influenced this trend.

The Allred scoring system was not the most ideal scoring system. The percentage of positive cells had very broad ranges, which made deciding the score on some cores difficult. For future directions, a different scoring system, such as the quick score, could be used. A quick score is done by scoring the positively stained cells and the intensity and multiplying them together, with the maximum score being 300. The percent positive score is broken down by the following: score of 0= <10% PC, score of 1+= 10-25% PC, score of 2+=25-50% PC, score of 3+= 50-75% PC, score of 4+=>75% PC. The intensity is scored as follows: score of 1= weak staining, 2= moderate staining, 3= strong staining.

Tumor grades are determined by pathologists, and are not standardized. This could have influenced the results, because the cores on the slide were graded by several pathologists. For future directions, it would be ideal if all of the tumor samples were re-graded by a single pathologist.

Between all of the tumor types scored, none showed statistical significance when compared to tumor grade. Statistical significance is achieved when the p-value is less than 0.05. As stated in the results section, each statistical test (ANOVA and Tukey Kramer) yielded p-values higher than 0.05, meaning there is no confirmed statistical significance. We cannot be confident in stating that there is no correlation between C5aR expression and tumor grade for all of the tumor types, because of the small sample sizes per each tumor type. Additionally, the ovary control sample size was one, and ideally there would be multiple. To reveal more confident conclusions, the study could be done again with overall higher sample sizes.

Past studies have used immunohistochemistry to observe C5aR expression in breast cancer. Imamura et. al. used a scoring system from 0-3, based on the amount of positively stained nuclei. They discovered that tumor samples that had expression of C5aR on tumor cell membranes correlated with tumor progression. These results were similar to what we predicted. Imamura et. al. had a sample size of 171, whereas the sample sizes in this study were small. Perhaps if the ovarian cancer sample sizes were larger, the results would be similar.

In the future, there are other ways that could be considered to observe C5aR expression in tumor samples including a western blot using patient biopsy samples. Additionally, an invasion and migration assay can be done with an ovarian cancer cell line that is treated with the C5aR antagonist PMX53. As a control, the assay should be done with untreated cells in addition to a cell line that has already undergone this process, such as a human bile duct cancer cell lines MBC and HuCCT1 (Nitta, et. al., 2013). Nitta et. al. discovered that C5aR expression correlated with an increase in invasion and migration in human bile duct cancer cell lines. We expect that the results for the ovarian cancer cell line will be similar.

To conclude, we cannot confidently state that there is a correlation between C5aR expression and ovarian cancer progression.

References:

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