Lack of FAS receptor expression in colorectal cancer patients correlates with high TNM staging

Sebastiaan N.J. Laan[1]
Supervisor: Dr. Peter J.K. Kuppen[1]
[1]*LUMC, Department of Surgery, Leiden University Medical Center*

Abstract
Cancer cells often inhibit the expression of FAS to evade T-cell mediated apoptosis upon HLA-mediated recognition. In this study, we aim to find the prognostic value of FAS expression, next to HLA expression, in colorectal cancer (CRC) patients. Colorectal tissue was immunohistochemically stained for the FAS and HLA receptors. Results show that there are significantly more patients with no FAS and HLA expressions in higher stages corresponding with tumor node metastasis (TNM) than in lower stages. Next to TNM stage, the loss of expression of FAS or of both FAS and HLA turns out to have no additional prognostic value.

Introduction
Cancer is the second leading cause of death globally and is responsible for nearly 1 in every 6 deaths (1). Normal cells can transform into cancer cells through the accumulation of genetic and epigenetic aberrations (2). Generally, it takes many years for enough of these aberrations to accumulate in order for a healthy cell to become cancerous. These aberrations can cause the proteins to become dysfunctional, overexpressed or lost, causing tumor invasiveness and metastases. One receptor that has been found to be aberrantly expressed in cancer is the Fas cell surface death receptor (FAS) (3). FAS is expressed by all nucleated cells and can bind to FAS-ligand (4). FASL is expressed on most immune cells, especially T-cells and NK cells. When cytotoxic T-cells bind to their target cells, FASL is up-regulated. FASL, in turn, binds to the FAS on the target cell and induces the apoptotic pathway. FAS can induce apoptosis through the caspase cascade and through the release of perforins. Cancer cells have been found to downregulate FAS to evade apoptosis (5,6). Conversely, the upregulation of FASL on tumors cells may cause apoptosis in cytotoxic T-cells as these cells are sensitive to FASL mediated apoptosis (7). FASL has two forms, one membrane bound, the other secreted. Membrane-bound FASL (mFASL) causes apoptosis but the secreted form (sFASL) has tumor-promoting capabilities *in vivo* (8). Another receptor of interest in CRC is human leukocyte antigen (HLA) class 1, which is pivotal in immunological recognition and elimination of aberrant cells (9,10). Some claim that HLA class 1 has no effect on survival (11), while others claim there is an effect (12).
Expression of FAS, FASL and HLA class 1 have been previously studied in CRC (13,14), although the function of FAS in cancers is not entirely clear (15). Therefore, this research aims to find the prognostic value of the FAS receptor and the HLA class 1 receptor in CRC patients. We expect to find a decrease in cancer-specific survival (CSS) in CRC patients with no FAS expression. Furthermore, we expect that the lack of HLA in combination with lack of FAS expression will further decrease CSS.

Materials and methods
study population
Patient samples were collected from the Dutch TME trial (16). The tumor staging was categorized with the tumor node metastasis (TNM) classification (17)(18). Three cores of 2 mm from each tumor sample were taken and included in tissue microarrays (TMA) (19). Enough formalin-fixed paraffin-embedded material was on hand for 495 patients. Normal colon tissue cores were also included in the study as controls. After removing patients with less than 2 tumor tissue cores available for analysis, 367 patients remained for full analysis.

Immunohistochemistry and microscopic analysis
TMAs were immunohistochemically stained for the FAS receptor (anti-FAS antibody ab2437, Abcam, UK, and envision conjugated with HRP, Dako, UK) microscopic analyses were independently performed by two observers (with good agreement: Kappa values = 0.77 and 0.84 for respectively % + intensity or % only of stained cells). This indicates that there was substantial agreement between the independent observers (21). Staining intensity of FAS was categorized: no staining, low staining, medium staining and high staining. HLA class 1 expression status was previously recorded (20).

Statistical Analysis

All analyses were performed with statistical software SPSS (version 23.0 for Windows, SPSS Inc, Chicago, USA). The Chi-squared test was performed on FAS categories and the combination of FAS and HLA expression to analyze possible associations between the groups and TNM staging. CSS since surgery was defined as time of surgery until relapse of cancer or death by disease. Kaplan-Meier analysis was performed to analyze the CSS since surgery and the Log-rank test was used for comparison between the two groups. Cox regression analysis was performed for multivariate analyses. Corrections were made for randomization, sex, age at randomization, TNM stage and Differentiation.

Results

FAS expression

Figure 1 shows representative images of the immunohistochemical staining for the FAS receptor in brown on CRC tissue (figure B-E) and healthy colorectal tissue (figure 1A). Stroma was stained in all cores. Tumor cells were easily identified as large unorganized structures within the core. The cores showed a variation of staining intensity and the various categories are shown in increasing intensity in figure B, C, D, and E respectively. The hematoxylin staining can be seen in purple/ blue.

FAS expression on CRC

To evaluate the prognostic value of FAS expression in CRC, a Kaplan-Meier analysis was performed on the entire cohort (figure 2A). The result shows that there is a significant correlation between a decrease in CSS since surgery and loss of FAS receptor expression. The log-rank test performed to evaluate the difference in survival curves resulted in a p-value of 0.018. Univariate analysis performed on the FAS expression resulted in a hazard ratio (HR) of 0.64 (95% CI 0.44-0.93, p-value = 0.019). The cohort was also corrected for a number of factors in a multivariate analysis. The cohort was corrected for age, sex, TNM staging, randomization, and differentiation. This resulted in a adjusted HR of 0.94 (95% CI 0.64-1.40, p-value = 0.772). It is evident that the expression of FAS does not hold any prognostic value. Further multivariate analysis was performed without correcting for the TNM staging. The HR without TNM staging was 0.71 (95% CI 0.49-1.05, p-value = 0.085), which is a trend.

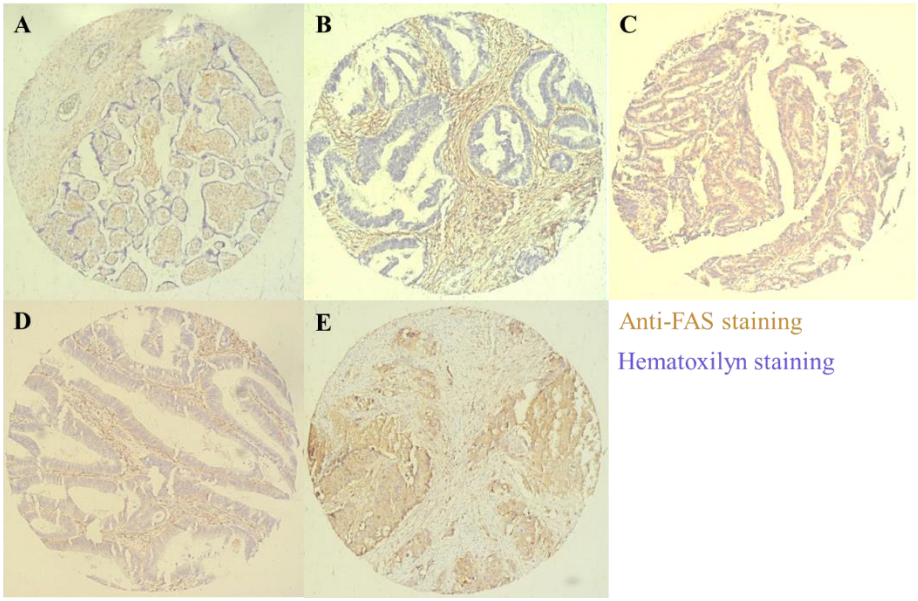

Figure 1. Representative examples of immunohistochemical staining for the FAS intensity staining.

Images from the 4 different FAS staining intensities. A healthy colorectal tissue serving as control is shown **(A)**. All tumor cores were categorized in four categories. Tumor cells that showed no staining **(B),** Tumor cells that were categorized as low intensity staining **(C)**, Medium intensity staining **(D)**, and high intensity staining **(E).**

Combination of FAS expression and HLA class 1 expression.

A combination of patients that lack FAS expression and HLA expression was compared to the patients that expressed some level of either receptor with a Kaplan-Meier (figure 2B). The patients without expression show a stronger correlation with CSS since surgery when compared to patients that have either mild, normal or strong expression of HLA and/or FAS (log-rank p-value = 0.001). Univariate analysis resulted in a HR of 0.36 (95% CI 0.20-0.66, p-value = 0.001). The adjusted HR after correction was 0.94 (95% CI 0.46-1.94, p-value = 0.87). Similarly to the FAS expression, the combination of HLA and FAS expression does not hold any prognostic value in CRC patients. The multivariate analysis was also performed without TNM staging for the combination of FAS and HLA. This resulted in a adjusted HR of 0.40 (95% CI 0.22-0.74, p-value = 0.04).

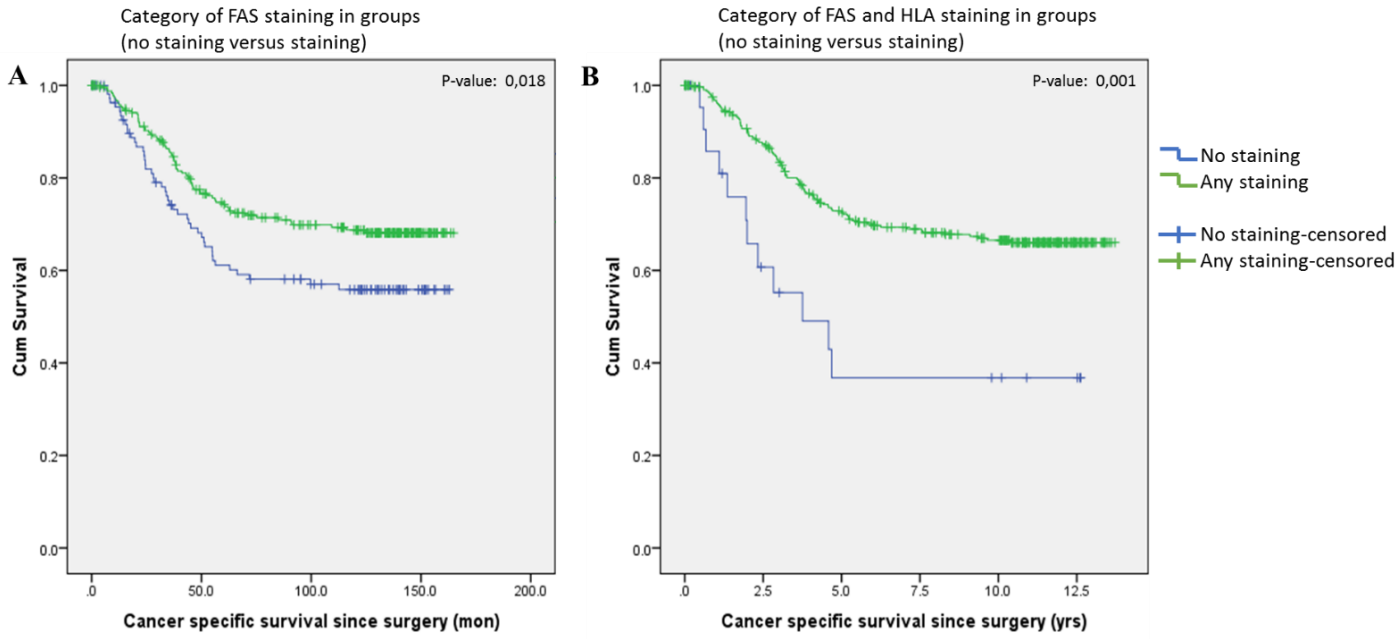

Figure 2. Kaplan-Meier survival analysis stratified for intensity of receptors.
Result of the Kaplan-Meier analyses for the cancer specific survival since surgery. Analysis was performed on the 367 eligible colorectal cancer patients. **(A)** FAS staining intensity was further categorized into two categories: no staining versus any staining (low staining, medium staining and high staining. **(B)** FAS staining intensity in combination with HLA class 1 expression. FAS was categorized in the same groups as mentioned before. In combination the no staining group consisted of patients that have no HLA expression as well.

Finally, A Pearson Chi-square test was performed to identify possible associations between FAS and/ or HLA expression and TNM staging. There was a difference between the TNM staging and the percentage of patients with no FAS expression (a trend, $p = 0.076$). For patients with no FAS and no HLA expression the difference was significant ($p = 0.001$).

Discussion

In this study, we aimed to determine the prognostic value of the FAS receptor in colorectal cancer. There was substantial agreement between the two observers, showing that the data are valid. The small discrepancy was likely caused by the difficulty of discerning the intensity of the FAS staining and the large amount of tissue artefacts in the samples. Our data showed that patients with no FAS receptor expression had a worse CSS since surgery when compared to patients that did express FAS. Furthermore, the lack of both HLA class 1 and FAS expression resulted in a worse CSS since surgery than the FAS receptor alone. However, the prognostic value was lost after correction in multivariate analysis for age, sex, TNM staging, randomization, and differentiation. The loss of prognostic value can be explained by the fact that the expression of FAS was associated with the TNM staging. Patients without FAS receptors expression were more likely to have a higher TNM staging. The combination of FAS and HLA show the same trend. The multivariate analysis without correcting for TNM staging gave p values ($p = 0.085$) indicating a trend for the lack of FAS expression and ($p = 0.04$) for the lack of both FAS and HLA class 1. Thus, the TNM staging is such a strong prognostic marker that it nullifies the effect of FAS and HLA class 1 expression.

The expression of FAS or the combination of FAS and HLA class 1 does not have a significant prognostic value in CRC next to TNM stage. However, a clear trend was observed between TNM staging and the expression of FAS and/ or HLA. Patients that had a higher TNM staging also were also more likely to not express FAS ($p=0.076$) by tumor cells and this effect was even stronger in combination with HLA ($p=0.001$). It can be concluded that patients with a higher TNM staging also have a higher chance of not expressing FAS and/or HLA. Apparently, there is a selection advantage for tumor cells with these receptors downregulated. Without, tumor cells can evade cell death and avoid recognition by cytolytic T-cells. This combination especially makes the cancer cells difficult to kill. Therefore, tumor expression of these receptors might have clinical prognostic value. A possible follow up study could look at the expression of FAS in CRC tissue from a different cohort. The same experiment can also be repeated on this cohort testing the tissue of the patients treated with both TME and radiotherapy to see whether radiotherapy works better on patients with or without FAS expression.

Role of the student.

Bas Laan was an undergraduate student working under the supervision of Peter Kuppen when the research in this report was performed. The idea was proposed by the supervisor and the staining largely done by Geeske Dekker. Imaging, scoring, analyzing and writing was performed by the student.

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