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Macrophage expression of tartrate-resistant acid phosphatase as a prognostic indicator in colon cancer

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Abstract

Recent research has indicated that separate populations of macrophages are associated with differing outcomes in cancer survival. In our study, we examine macrophage expression of tartrate resistant acid phosphatase (TRAP) and its effect on survival in colon cancer.

Immunohistochemical analysis on colorectal adenocarcinomas confirmed macrophage expression of TRAP. Co-localization of TRAP with CD68, a pan-macrophage marker, revealed that TRAP is present in some but not all subpopulations of macrophages. Further co-localization of TRAP with CD163, an M2 marker, revealed that TRAP is expressed by both M2 and non-M2 macrophages.

TRAP expression was then measured using the AQUA method of quantitative immunofluorescence in a tissue microarray consisting of 233 colorectal cancer patients seen at Yale-New Haven Hospital. Survival analysis revealed that patients with high TRAP expression have a 22% increase in 5-year survival (uncorrected log rank $p=0.025$) and a 47% risk reduction for disease specific death ($p=0.02$). This finding was validated in a second cohort of older cases consisting of 505 colorectal cancer patients. Patients with high TRAP expression in the validation set had a 19% increase in 5-year survival (log rank $p=0.0041$) and a 52% risk reduction of death ($p=0.0019$). These results provide evidence that macrophage expression of TRAP is associated with improved outcome, and implicates TRAP as a potential biomarker in colon cancer.

Keywords

Tartrate-resistant acid phosphatase; M1; M2; macrophage; colon cancer; biomarker

Introduction

Despite large improvements in treatment, the current 5-year survival rates for colon cancer patients still range from 10% to 90% as a function of stage. Although many biomarkers have been reported, standard of care for colon cancer is still largely limited to assessment of TNM-based stage. Given the wide range of survival outcomes, the development of accurate prognostic markers has been an area of intensive research.

Recent work has described the evaluation of the immune response as a prognostic indicator in colorectal cancer (CRC). Multiple studies have suggested that infiltration with immune

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cells such as macrophages, CD4+ and CD8+ T cells, and dendritic cells is associated with improved survival outcome (Forssell et al., 2007; Galon et al., 2006; Mlecnik et al., 2011; Nagorsen et al., 2007). In particular, macrophage infiltration confers a significant survival advantage in patients with CRC (Forssell et al., 2007). However, additional research has also implicated tumor-infiltrating macrophages in the role of tumor growth and development, with increased macrophage infiltration associated with significantly poorer clinical outcome in breast (Goede et al., 1999; Robinson et al., 2009), lung (Koukourakis et al., 1998), bladder (Hanada et al., 2000), cervical cancer (Fujimoto et al., 2000), and Hodgkin's lymphoma (Steidl et al., 2010). These seemingly contradictory findings are largely due to the divergent actions of macrophages in differing microenvironments. Macrophages may exhibit tumoricidal effects through the production of toxic intermediates such as nitric oxide (NO) or reactive oxygen species (ROS) (Mantovani et al., 2004). However, macrophages are also able to exhibit tumorigenic effects through the production of growth factors (Robinson et al., 2009), promotion of angiogenesis (Goede et al., 1999), and downregulation of inflammatory reactions (Mantovani et al., 2007; Mantovani et al., 2004).

These divergent actions have resulted in the identification of two subpopulations of macrophages, each with its own response to the tumor microenvironment. M1, or "classically activated" macrophages, are primarily involved in tissue destruction and microbial killing via the production of cytotoxic intermediates and activation of the Th1 type immune response (Mantovani et al., 2007; Mantovani et al., 2004). M2, or "alternatively activated" macrophages, consist of all non-classically activated macrophages, and are primarily involved in tissue remodeling, angiogenesis, and anti-inflammatory responses (Mantovani et al., 2007; Mantovani et al., 2004). Thus, it has been proposed that while M1 macrophages promote tumor killing, M2 macrophages promote tumor growth (Mantovani et al., 2004; Talmadge et al., 2007). Indeed, multiple studies have shown that distinct subsets of macrophages are present in different tumor microenvironments (Hakansson et al., 1997; Hauptmann et al., 1994; Movahedi et al., 2010), and the distribution of M1 versus M2 activity has significant effects on outcome (Heys et al., 2012; Medrek et al., 2012). In CRC, the M1/M2 distinction has been less clear in guiding prognosis, as while studies have also shown improved survival with increased M1 macrophage infiltration, there is also improved survival with increased M2 macrophage infiltration as well (Edin et al., 2012; Nagorsen et al., 2007).

In this study, we investigate macrophage expression of tartrate-resistant acid phosphatase (TRAP) as a potential biomarker in colon cancer outcome. TRAP is a metalloprotease that catalyzes hydrolysis of phosphate esters (Oddie et al., 2000). It is highly expressed in osteoclasts and was first discovered for its importance in bone resorption (Janckila and Yam, 2009). Clinically, TRAP has been used as a biomarker for growth and bone turnover and hairy cell leukemia (Janckila and Yam, 2009; Lamp and Drexler, 2000). However, in addition to its roles in skeletal development, TRAP is highly expressed in activated macrophages and plays an important function in innate immunity. TRAP has been implicated in catalyzing the generation of ROS (Oddie et al., 2000), and it has been observed that macrophages overexpressing TRAP display increased superoxide production and bacterial killing (Raisanen et al., 2005). In addition, certain substrates of TRAP have been shown to mediate Th1 type immunity, resulting in macrophage production of cytokines

typical of M1 activity (Ashkar et al., 2000; Hayman, 2008). Interestingly, recent research has suggested that tumor expression of TRAP is a negative prognostic marker in cancers with bone metastasis (Honig et al., 2006) and melanoma (Scott et al., 2011). However, we know of no examples of examination of TRAP expression as a prognostic indicator in the context of immune mediated responses to tumor invasion.

Materials and Methods

Tissue Microarrays and Patient Cohorts

Tissue microarrays (TMAs) for two separate and independent cohorts were constructed at the Yale University TMA facility (New Haven, CT) with formalin-fixed, paraffin-embedded tumor samples, as described previously (Rimm et al., 2001a; Rimm et al., 2001b). The more recent set contained 276 primary colorectal carcinomas from patients who were treated at Yale New Haven Hospital in New Haven, CT from 2000–2005. The earlier set contained 629 primary colorectal carcinomas from patients who were treated at Yale New Haven Hospital in New Haven, CT from 1970–1981. All follow-up information on the patients was obtained from the Yale New Haven Tumor Registry, the Yale-New Haven Hospital medical records and the Connecticut Death Records. Demographic and clinical information on each cohort is summarized in Table 1. For the purposes of this study, the newer cohort (YTMA 221) was used as the training set and the older cohort (YTMA 8) was used as the validation set. Disease-specific death was used to measure survival in the validation set, but due to lack of information on cause of death in the training set, alive-dead status was used to measure survival in the training set. In order to assess whether there were any significant differences between the training and validation sets, we performed two-sample proportions and t-tests on each of the demographic and clinical characteristics. There were no significant differences in the proportion of females ($p=0.28$) and the proportion of patients with Stage I ($p=0.56$), Stage II ($p=0.82$), Stage III ($p=0.62$) and Stage IV ($p=0.08$) colorectal carcinomas in each cohort. A significant difference was found in the mean patient age of each cohort ($p=0.03$); however, the mean patient age between the two cohorts differed by only about two years (training: 69.6 years; validation: 67.5 years). In addition, there were significant differences in the proportion of patients with well, moderately, and poorly differentiated colorectal carcinomas between the two cohorts ($p<0.01$). However, a survival analysis of the two cohorts revealed no significant differences in overall survival ($p=0.99$). These results indicate that the two cohorts were reasonably similar in major demographic and clinical characteristics.

TMAs were constructed in two-fold redundancy for each cohort. Average redundancy in analysis was approximately 60%. A control (index) TMA containing 34 primary colorectal carcinomas from patients treated at Yale New Haven Hospital from 1970–1981 was used for run to run standardization.

Immunohistochemistry and Immunofluorescence

Immunohistochemical visualization of TRAP or CD68 was performed using a diaminobenzidine (DAB) staining protocol on serial index arrays. Slides were first deparaffinized by baking at 60°C for 30 minutes followed by 2× xylene treatment for 20 min

each. Antigen retrieval was performed by pressure cooking with citrate buffer pH 6 at 97°C for 20 min. Slides were then permeabilized in 0.3% H₂O₂ in methanol for 30 min in the dark, followed by preincubation with 0.3% bovine serum albumin (BSA) in 0.1 M Tris buffered saline (TBS, pH 8) for 30 min at room temperature. A primary antibody against CD68 (rabbit polyclonal ab125047; 1:750; Abcam, Cambridge, MA, USA) or TRAP (mouse monoclonal ab49507; 1:100; Abcam) diluted in 0.3% BSA/TBS was applied overnight at 4°C. After washing, slides were incubated with either an anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (EnVision; DaKo, Carpinteria, CA, USA) for CD68 or TRAP, respectively. The detection reaction was developed with DAB (DAB Enhancer; DaKo) for 5 min, then washed and counterstained with Tacha hematoxylin (Biocare Medical, Concord, CA, USA) for 1 min. Slides were then dehydrated in ethanol and mounted with xylene for 5 min, followed by coverslipping with Cytoseal 60 (Thermo Scientific, Waltham, MA, USA).

Immunofluorescent visualization of TRAP and CD68 co-staining was also performed on index arrays. Slides were deparaffinized and preincubated using the same procedures above. However, CD68 (rabbit polyclonal ab125047; 1:1000; Abcam) and TRAP (mouse monoclonal ab49507; 1:100; Abcam) antibodies were both diluted in 0.3% BSA/TBS during the primary incubation. Slides were then washed and incubated with a secondary antibody conjugated to a Cy3 fluorophore (Alexa 546 goat anti-rabbit; 1:100; Molecular Probes, Grand Island, NY, USA) diluted in an anti-mouse antibody conjugated to horseradish peroxidase (EnVision; DaKo). To allow visualization of TRAP, slides were then washed and incubated with Cy5 conjugated tyramide (1:50; PerkinElmer, Hopkington, MA, USA) for 10 min. Coverslipping was performed using Prolong Gold mixed with DAPI (Molecular Probes).

Multiplexing of TRAP with CD163 was similarly performed on test arrays using the same deparaffinization and preincubation procedures. TRAP and CD163 (mouse monoclonal CD163-L-U; 1:50; Novocastra) were diluted in 0.3% BSA/TBS for 30 min during the primary incubation. Slides were washed and first incubated with an anti-IgG1 secondary antibody conjugated to HRP (goat anti-mouse monoclonal 18-4015-82; 1:100; eBioscience), and then incubated with Cy3 conjugated tyramide (1:50; PerkinElmer, Hopkington, MA, USA) for 10 min. to allow visualization of CD163. Slides were then quenched with 0.5 mM benzoic hydrazide solution with 0.5% H₂O₂, followed by incubation with an anti-IgG2b secondary antibody conjugated to HRP (goat anti-mouse monoclonal ab97250; 1:100; Abcam). To allow visualization of TRAP, slides were again washed and incubated with Cy5 conjugated tyramide.

Immunofluorescence of TMAs for AQUA analysis followed the same procedures as above. However, an antibody against pancytokeratin (rabbit polyclonal; 1:100; Dako) was used instead of CD68 during the primary incubation to identify epithelium. Serial sections of an index array were also stained alongside each cohort to assess the inter-assay reproducibility.

Image Acquisition

Image acquisition for immunohistochemical arrays was performed using a ScanScope microscope (Aperio, Vista, CA, USA). Automated image capture of immunofluorescence

was performed using the HistoRX PM2000 device, as described previously (Moeder et al., 2009). Images of each histospot on the array were captured. Nuclear, CD68 or cytokeratin, and TRAP staining were visualized with DAPI, Cy3, and Cy5 channels, respectively.

Quantitative Immunofluorescence (QIF)

The AQUA method of QIF allows quantitative measurements of protein levels in subcellular compartments, as described previously (Camp et al., 2002; Moeder et al., 2009). Briefly, a binary tumor mask is generated using cytokeratin staining as an indicator of tumor epithelial cells, and a cellular mask is generated from dilation of nuclei created from the DAPI staining. The tumor mask is then subtracted from the DAPI generated tissue and cellular mask, resulting in a new compartment that represents only the stromal tissue in each histospot (Figure 1). The signal intensity within this compartment is then divided by the area of this “stromal mask” in order to generate a TRAP AQUA score. These AQUA scores are then used for selection of cut-off points and subsequent analysis, as described below.

Statistical Analysis

For both cohorts, TRAP AQUA scores from two independent cores were averaged for final analysis. Optimal cut-off points for the training set were determined by X-tile, as described previously (Camp et al., 2004). Kaplan-Meier curves, univariate, and multivariate Cox proportional hazards ratios were then generated using JMP 9 (SAS Inst, Glastonbury, CT, USA). Pearson's correlation coefficient (R^2) and linear regressions between near-serial sections of the index array were used to assess inter-array reproducibility, and to normalize the validation set to the training set. Following normalization, the cut-off point generated from the training set was applied to the validation set to generate a second Kaplan-Meier curve. Additional statistical analyses, including a univariate and multivariate Cox proportional hazards analysis, were conducted using JMP 9.

Results

Differential TRAP Expression Revealing Two Populations of Macrophages

We performed immunohistochemical staining on serial arrays of colorectal carcinomas to determine which cell types expressed TRAP. Positive TRAP expression was observed primarily in extraepithelial tissue in cells with macrophage morphology. Figure 2a illustrates a representative example of a macrophage expressing TRAP. To determine whether TRAP is expressed in all macrophages, or whether it can distinguish differing sub-populations of macrophages, we assessed co-localization of TRAP in cells that were also expressing the common macrophage marker CD68. An example of routine identification of macrophages by immunostaining with CD68 is shown in Figure 2b. Figures 2c–e illustrate the results of immunofluorescent colocalization. As expected, TRAP staining (green) was observed primarily in stromal tissue, with several positive cells displaying macrophage morphology (Figure 2c). In addition, CD68 expression (red) displayed a similar staining pattern in the stromal tissue (Figure 2d). Merged images however revealed that TRAP expression was not completely identical to CD68 expression. Although many cells demonstrated co-localized TRAP and CD68 expression, there were also several CD68+ cells that had no TRAP expression (Figure 2e). No CD68– and TRAP+ cells were observed. This observation

suggests that at least two populations of macrophages exist, those that express both CD68 and TRAP, and those that express CD68 but do not express TRAP.

To determine if TRAP expression is associated with a specific macrophage subtype, we assessed co-localization of TRAP with CD163, an M2 marker used commonly in the literature (Figure 3) (Komohara et al., 2006). Figure 3c shows co-localization of TRAP with CD163, indicating M2 expression of TRAP. However, there is also evidence of TRAP+ and CD163- cells (Figure 3f), indicating that TRAP is not exclusively expressed by M2 macrophages.

TRAP Expression and its Association with Favorable Outcome in Colorectal Cancer

Macrophages and inflammatory cells are considered to be stromal components that can affect the behavior of the adjacent tumor (Goede et al., 1999; Mantovani et al., 2007; Mantovani et al., 2004). Specifically M1 macrophages have been associated with tumor suppressive activity while M2 macrophages are tumor promoting (Mantovani et al., 2004; Talmadge et al., 2007). TRAP appears to be expressed in only a subset of macrophages, although it has not been associated with a subtype. Since macrophages are localized to stroma, AQUA scores for TRAP were generated by measurement of signal intensity in the stromal compartment. The AQUA scores were averaged between two independent cores. Inter-array reproducibility was also measured by staining serial sections of an index array with colorectal carcinomas from a small sub-group of control patients. The Pearson's R^2 value for the index arrays between the training and validation set was 0.96. The high Pearson's value indicates not only good experimental reproducibility, but also suggests relative homogeneity of TRAP expression in tumor tissue.

Measurement of expression of stromal TRAP in the newer colon cancer cohort showed a rightward skewed bell-shaped distribution. Since there was no obvious cut-point in the distribution, we used a statistical method called X-tile, which has been previously described (Camp et al., 2004), to define an optimal cut-point on the basis of overall survival. The optimal cut-point is shown as an inset in figure 4a and the resulting survival curve is shown in Figure 4b. The optimal cut-off point was determined to be an AQUA score of 2281 (uncorrected log-rank $p=0.025$), with 33.5% of patients in this cohort belonging to the "high" group (Figure 4a). A Kaplan-Meier curve was then generated to illustrate the differences in survival between the two groups (Figure 4b). Patients with high TRAP expression had a 22% increase in 5-year survival from patients with low TRAP expression (from 52.0% to 71.2%; uncorrected log-rank $p=0.025$).

Since the optimal cut-point was determined from all possible cut-points, correction is required for multiple testing. Rather than use a statistical approach, we selected a second, older, but larger cohort of colon cancer patients to serve as a validation set. The cut-off point was then applied to the validation set in order to determine if TRAP expression is significantly associated with favorable outcome. In the validation set, 15.8% of patients had TRAP expression higher than the normalized applied cut-off (Figure 5a). A Kaplan-Meier curve demonstrated a significant increase in survival for patients with high TRAP expression ($p = 0.0041$; Figure 5b). Overall, patients with high TRAP expression in the validation set had a 19% increase in 5-year survival (from 58.8% to 77.7%), confirming the

initial findings in the training set. These results suggest that TRAP expression is correlated with favorable outcome in colorectal cancer.

Univariate and Multivariate Analyses Reveal TRAP Expression as Independent of Age, Gender, and Grade in Survival

We performed univariate and multivariate Cox proportional hazards analyses to determine the prognostic value of TRAP while also controlling for potential confounding factors. In the new cohort, patients with high TRAP expression experienced a 47% risk reduction of death compared to patients with low TRAP expression (hazards ratio 0.53, $p=0.02$; Table 2a). In the older cohort, patients with high TRAP expression experienced a 52% risk reduction of colorectal cancer death compared to patients with low TRAP expression (hazards ratio 0.48, $p=0.0019$; Table 2a). When controlling for other prognostic factors such as age, gender, and grade, high TRAP expression was associated with a 46% reduction in death in the training set (hazards ratio 0.54, $p=0.04$) and a 51% reduction in colorectal cancer death in the validation set (hazards ratio 0.49, $p=0.05$; Table 2b). However, high TRAP expression was not significantly associated with decreased risk when stage was included in the analysis for both the training and validation sets ($p=0.06$ and $p=0.12$, respectively). Since low stage patients generally have good outcome, we performed COX multivariate proportional hazards ratio analysis to determine the prognostic value of TRAP restricted to patients with Stages III and IV colorectal cancer only (Table 3). In YTMA221 (new set), high TRAP expression was associated with a 66% reduction in death, independent of stage ($p=0.0036$). In YTMA8 (old set), high TRAP expression was associated with a 50% reduction in death, independent of stage ($p=0.026$). In these analyses, age, gender, and grade were not significantly associated with risk of death (Table 2b).

Discussion

TRAP or ACP5 has not yet been well characterized with respect to its prognostic value, in spite of interesting mechanistic observations on the role of TRAP in tumor invasion (Hayman, 2008) and in the host immune response to tumor invasion (Mantovani et al., 2004; Oddie et al., 2000; Raisanen et al., 2005). We found TRAP expression in the stroma of tumor tissue, and showed morphological evidence that the expression was localized to a subset of macrophages using immunohistochemical stains. Furthermore, co-localization with CD68, a pan-macrophage biomarker, revealed that TRAP was not present in all macrophages. Further co-localization of TRAP with CD163 revealed expression of TRAP in both M2 and non-M2 macrophages. There is a great deal of evidence in the literature that CD68+ macrophages are associated with improved survival (Algars et al., 2012). We were interested in whether certain subgroups of CD68+ cells (i.e. TRAP+ cells) are also associated with improved patient outcome. Survival analyses revealed that patients with high TRAP expression was significantly associated with improved 5-year survival rates and decreased risk of death. These results indicate TRAP's potential use as a biomarker for favorable outcome in colon cancer, and add further complexity to the M1/M2 distinction in CRC prognosis.

To our knowledge, this is the first study to assess TRAP as a biomarker for macrophage activity in cancer. Previous studies have shown that TRAP is associated with poor outcome when expressed by tumor epithelial cells in melanoma, breast, and ovarian cancer (Honig et al., 2006; Scott et al., 2011), although the mechanism of action has not yet been elucidated. In addition, while previous research has confirmed TRAP's importance in the clearance of pathogens (Raisanen et al., 2005), none have yet looked at TRAP's tumoricidal potential. It is believed that TRAP's importance in innate immunity derives from its ability to catalyze the production of ROS (Jankila and Yam, 2009; Oddie et al., 2000; Raisanen et al., 2005). Also, some evidence exists that substrates for TRAP are essential for microbial defense (Ashkar et al., 2000; Hayman, 2008). However, TRAP expression by M2 macrophages may also indicate additional roles of TRAP that we cannot yet define.

The results of this study are consistent with existing research on M1/M2 macrophages in CRC. Many clinical studies have suggested that M1 macrophages are associated with improved outcome (Heys et al., 2012), while M2 macrophages are associated with worse outcome (Heys et al., 2012; Medrek et al., 2012). However, there is evidence that M2 macrophages are associated with increased survival in CRC as well, specifically see work by Edin and Nagorsen. (Edin et al., 2012; Nagorsen et al., 2007). Some researchers have postulated that the anti-tumorigenic effect of M1 macrophages may dominate over the tumor promoting effect of M2 macrophages (Edin et al., 2012). However, it is also possible that M2 macrophages may also have differing effects on tumorigenesis in CRC compared to other cancer types. In particular, the expression of TRAP in both M2 and non-M2 macrophages may indicate an altered M2 profile in CRC, such that both TRAP and M2 expression are associated with improved prognosis. Alternatively, although CD163 has been used to mark M2 macrophages, there is also evidence for some plasticity of monocyte differentiation, and the presence of diverse macrophage phenotypes within the M1 and M2 designations (Mantovani et al., 2004). Thus, M1 and M2 activation may represent a spectrum rather than a simple dichotomy of macrophage function (Mantovani et al., 2004). Our data supports the possibility that TRAP represents an alternative biomarker, although it appears not to exclusively segregate with M1 or M2 activation. In addition, because TRAP activity may have a direct effect on tumoricidal activity, further exploration of TRAP's mechanisms of action may open up avenues for immune-mediated therapeutics.

There are a number of limitations in this study. We focused on TRAP's effect on survival outcome in colorectal cancer and used primarily morphological assessment of expression to investigate which subtypes of macrophage populations express TRAP. Given TRAP's expression in M2 macrophages, it is unclear what is TRAP's role in tumorigenesis. More research on both TRAP's functions and the M1/M2 profile would thus be beneficial in the future for better understanding of the functions of this protein. In our study, we did not observe any CD68⁻ TRAP⁺ cells, but the presence of non-macrophage cells with positive TRAP expression cannot be discounted, as dendritic cells have been known to express TRAP (Hayman, 2008). Thus, it is possible that TRAP's association with improved survival may also be due to expression by other immune cells.

A second limitation of the work is that it was performed entirely on tissue microarray cohorts. While this is now a common approach, future studies will be required to determine

if conventional slide analysis of TRAP is consistent with the observations we have made on the tissue microarrays. In addition, further validation of TRAP's prognostic role in larger, multi-institutional cohorts should be considered.

In conclusion, this study demonstrates a novel application of TRAP in the prognosis of colorectal cancer, and provides further evidence for subclasses of macrophages with differential roles in the tumor microenvironment.

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Fig. 1.

a–f Development of a Stromal Compartment in AQUA. **(a)** Nuclei stained by DAPI are dilated into a binary mask to create a **(c)** DAPI mask. As previously described, **(b)** cytokeratin staining is used to generate a binary mask of the epithelial compartment, called the **(d)** tumor mask. The tumor mask is subsequently subtracted from the DAPI mask to generate the **(e)** stromal mask. **(f)** Staining of the target TRAP (white) within the stromal mask (blue)

Fig. 2.
a–e Immunohistochemical and immunofluorescent photomicrographs of TRAP and CD68 expression on colorectal carcinoma tissue cores. Hematoxylin and positive diaminobenzidine (DAB) staining reveals macrophage expression of TRAP (**a**) and confirms CD68 as a marker for macrophages (**b**). (**c**) Immunofluorescent staining of TRAP (green) with DAPI nuclear staining (blue). (**d**) Immunofluorescent staining of the macrophage marker CD68 (red) with DAPI nuclear staining (blue). (**e**) Merged image of TRAP and CD68 staining indicating at least two populations of macrophages. Red indicates a CD68+ and TRAP– macrophage, while yellow indicates a CD68+ and TRAP+ macrophage.

Fig. 3.
a–f Immunofluorescent photomicrographs of TRAP and CD163 expression on colorectal carcinoma tissue cores. (a, d) Immunofluorescent staining of TRAP (green) with DAPI nuclear staining (blue). (b, e) Immunofluorescent staining of the M2 macrophage marker CD163 (red) with DAPI nuclear staining (blue). (c, f) Merged image of TRAP and CD163 staining indicating both co-localization of TRAP and CD163 (c) as well as TRAP+ and CD163– macrophages (f).

Fig. 4.
a–b (a) Histogram showing distribution of AQUA scores for stromal TRAP expression in the training set, divided into high and low populations. Inset shows the X-tile plot generated to determine the optimal cut-off point (arrow; see text for more details). (b) Kaplan-Meier curve of the training set showing differences in survival between patients with high and low TRAP expression using the generated cut-off point.

Fig. 5.
a–b (a) Histogram showing distribution of AQUA scores for stromal TRAP expression in the validation set, divided into high and low populations. The optimal cut-off point was previously generated from the training set. (b) Kaplan-Meier curve of the validation set showing differences in survival between patients with high and low TRAP expression using the generated cut-off point.

Table 1

Summary of clinical and pathological characteristics of colorectal carcinoma cohorts.

	New Set (YTMA-221)	Old Set (YTMA-8)
Total Number	233	505
25% Survival, mo	30 (alive-dead)	25 (disease-specific death)
Age		
Range	30–97	23–94
Mean	69.59	67.49
Median	72: 121 (51.9)	68: 256 (50.7)
	>72: 112 (48.1)	>68: 249 (49.3)
Sex		
Female	118 (50.6)	277 (54.9)
Male	111 (47.6)	228 (45.1)
Unknown	4 (1.7)	
Histologic Grade		
Well diff.	4 (1.7)	161 (31.9)
Moderately diff.	158 (67.8)	195 (38.6)
Poorly diff.	45 (19.3)	44 (8.7)
Unknown	26 (11.2)	105 (20.8)
Stage		
I	45 (19.3)	107 (21.2)
II	59 (25.3)	124 (24.6)
III	94 (40.3)	194 (38.4)
IV	35 (15.0)	53 (10.5)
Unknown		27 (5.3)

Table 2

a–b (a) Univariate analysis of patients with high and low TRAP expression. **(b)** Multivariate analysis of patients with high and low TRAP expression, age, sex and grade.

a. Univariate Analysis of TRAP Ratio		
	Hazard Ratio (95% CI)	P Value
New Set (YTMA-221)		
TRAP (High/low)	0.53 (0.29–0.91)	0.02
Old set (YTMA-8)		
TRAP (High/low)	0.48 (0.28–0.78)	0.0019

b. Multivariate Analysis of TRAP Ratio		
	Hazard Ratio (95% CI)	P Value
New Set (YTMA-221)		
Age (High/low)	0.77 (0.46–1.27)	0.30
Sex (F/M)	1.08 (0.65–1.79)	0.78
Grade (Poor/Well+Mod)*	1.80 (0.97–3.17)	0.06
TRAP (High/low)	0.54 (0.28–0.96)	0.04
Old Set (YTMA-8)		
Age (Above/low)	1.10 (0.69–1.76)	0.68
Sex (F/M)	0.83 (0.52–1.33)	0.43
Grade (Poor/Well)	1.65 (0.94–2.78)	0.08
TRAP (High/low)	0.49 (0.20–1.00)	0.05

* Due to the low number of patients with well differentiated carcinomas, patients with moderately and well differentiated carcinomas were combined together for grade analysis in the training set.

Table 3

Multivariate analysis of high and low TRAP expression and stage in Stage III and IV patients.

	Hazard Ratio (95% CI)	P Value
New Set (YTMA-221)		
TRAP (High/low)	0.34 (0.14–0.72)	0.0036
Stage (IV/III)	3.35 (1.84–6.03)	0.0001
Old Set (YTMA-8)		
TRAP (High/low)	0.50 (0.23–0.93)	0.0263
Stage (IV/III)	0.54 (0.31–0.87)	0.0105