NKG2D Ligand Expression in Human Colorectal Cancer Reveals Associations with Prognosis and Evidence for Immunoediting

Roger W. McGilvray,[1] Robert A. Eagle,[4] Nicholas F.S. Watson,[2] Ahmad Al-Attar,[1] Graham Ball,[3] Insiya Jafferji,[4] John Trowsdale,[4] and Lindy G. Durrant[1]

Abstract **Purpose:** NKG2D (natural killer group 2, member D) binds to cellular ligands of the MIC and ULBP/RAET family. These ligands have restricted expression in normal tissue, but are frequently expressed on primary tumors. The role of NKG2D ligands is thought to be important in carcinogenesis but its prognostic effect has not been investigated in such a large cohort.

Experimental Design: In our study, 462 primary colorectal tumors were screened for the expression of all MIC/ULBP/RAET proteins and NK cell infiltration. Tumor microarray technology was used for the purpose of this investigation.

Results: NKG2D ligands were expressed by the majority of colorectal tumors; however, the level of expression varied considerably. High expression of MIC (68 versus 56 months) or RAET1G (74 versus 62 months) showed improved patient survival. Tumors expressing high levels of MIC and RAET1G showed improved survival of 77 months over tumors that expressed high levels of one ligand or low levels of both. High-level expression of all ligands was frequent in tumor-node-metastasis stage I tumors, but became progressively less frequent in stages II, III, and IV tumors. Expression of MIC was correlated with NK cellular infiltration.

Conclusion: The observations presented are consistent with an immunoediting mechanism that selects tumor cells that have lost or reduced their expression of NKG2D ligands. The combination of MIC and tumor-node-metastasis stage was found to be the strongest predictor of survival, splitting patients into eight groups and suggesting prognostic value in clinical assessment. Of particular interest were stage I patients with low expression of MIC who had a similar survival to stage III patients, and may be candidates for adjuvant therapy. (Clin Cancer Res 2009;15(22):6993–7002)

NKG2D (natural killer group 2, member D) is a stimulatory receptor expressed on the surface of NK cells and subsets of T cells (1). It is unusual among activating receptors in binding to a diverse array of cellular ligands (2). Human NKG2D ligands comprise two members of the MIC (MHC class I–related chain) family and six members of the ULBP/RAET (UL16 binding protein, or retinoic acid early transcript) family (3–7). In mice, they include five members of the Rae1 (retinoic acid early inducible) family, the minor histocompatibility antigen H60, and Mult1 (murine ULBP-like transcript; refs. 8–10).

NKG2D ligand expression is generally absent from healthy tissues but can be induced on infection, and by cell stress stimuli. NKG2D ligands are also widely expressed on a variety of cancer cell lines, as well as primary solid tumors and leukemia (4, 11–14). The mechanisms regulating NKG2D ligand expression in cancer are not well understood, although activation of DNA damage response pathways have been implicated, as has the expression of the BCR/ABL oncogene (15–17).

In mouse models, it has been shown that tumor cell lines transfected with Rae1 are rejected *in vivo* via NKG2D-mediated immunity (18, 19). The recent generation of an NKG2D knockout mouse has provided the most convincing evidence to date for NKG2D involvement in antitumor immune responses (20). Using the knockout mice in conjunction with several different cancer models it became clear NKG2D interactions are variable between different types of cancer. For example, there was no increase in the incidence of methylcolanthrene-induced tumors in the knockout compared with wild-type; however, NKG2D deficiency was associated with increased incidence of

Authors' Affiliations: [1]Academic Division of Clinical Oncology, University of Nottingham, City Hospital Campus, [2]Section of Gastrointestinal Surgery, Queen's Medical Centre, [3]John Van Geest Research Centre, Nottingham Trent University, Clifton Campus, Nottingham, United Kingdom, and [4]Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrookes Hospital, Cambridge, United Kingdom
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Note: R.W. McGilvray and R.A Eagle contributed equally to this work.

Requests for reprints: Lindy Durrant, Academic Division of Clinical Oncology, University of Nottingham, City Hospital Campus, Nottingham, NG5 1PB, United Kingdom. Phone/Fax: 44-115-823-1863; E-mail: lindy.durrant@nottingham.ac.uk.

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Translational Relevance

The work presented here is the first comprehensive screen of NKG2D ligand expression on a large cohort of primary human colorectal cancers. Using tissue microarray technology, our analysis has shown that ligand expression is heterogeneous and seems to have a cooperative benefit towards improved prognosis. We describe the statistical analysis of ligand expression, highlighting those situations that show statistical significance to patient survival. Mathematical modeling has identified a group of at-risk patients that has not been previously described. This group of early stage cancer patients would likely benefit from adjuvant therapy in addition to the currently accepted regimen of surgical resection for stage I patients.

prostate adenocarcinomas and accelerated progression of Eμ-myc–induced lymphomas (20).

It is now widely accepted that tumors develop ways to evade anticancer immunity through a process termed immunoediting (21). A number of mechanisms have been proposed by which cancers could evade NKG2D-mediated immune responses. In some systems, persistent expression of NKG2D ligands can result in downregulation of NKG2D expression (22–24). It is also proposed that tumors may shed soluble NKG2D ligands, or secrete immunosuppressive cytokines such as transforming growth factor-β to downregulate NKG2D expression (25–27).

It is known that NKG2D ligands can be expressed independently of each other in human cell lines and primary tumors (4, 12, 28). It is also clear that different ligands can be expressed in response to different cancer-specific pathways. For example, in the cell line, K562, the BCR/ABL oncogene induced the expression of MICA but not ULBP1 and ULBP2 (16). NKG2D ligand expression was also heterogeneous between different tumors that arose in the knockout mouse. Prostate tumors arising in these mice had higher levels of NKG2D ligand expression than in wild-type mice. This suggests that tumor cells under selection switch off NKG2D ligand expression as part of an immunoediting process (20). A similar observation has been made in perforin-deficient mice (29).

Tissue microarray technology allows simultaneous immunohistochemical analysis of hundreds of tumor specimens for target protein expression (30). Data derived from these analyses can then be linked to clinicopathologic data so as to evaluate potential prognostic markers. In this article, we describe the analysis of the expression of all human NKG2D ligands using a large series of formalin-fixed, paraffin-embedded colorectal cancer tissue arrays, demonstrating that several findings from the NKG2D knockout mouse, such as heterogeneous NKG2D ligand expression and evidence for immunoediting, are also a feature of human disease. Our analysis identified the two strongest prognostic factors in colorectal cancer that retain independent significance as tumor-node-metastasis (TNM) stage and MIC expression. Using a mathematical prognostic model, these two factors indicated the presence of an at-risk group of TNM 1 patients who would potentially benefit from additional adjuvant therapy.

Materials and Methods

Patients and study design. The study population comprised a series of 462 consecutive patients undergoing elective surgical resection of a histologically proven sporadic primary colorectal cancer at the University Hospital, Nottingham, United Kingdom and has been reported on previously (31–35). Follow-up was calculated from time of resection of the original tumor with all surviving cases being censored for data analysis on December 31, 2003, this produced a median follow-up of 37 mo (range, 0-116) for all patients and 75 mo (range, 36-116) for survivors.

Adjuvant chemotherapy consisting of 5-fluorouracil and folinic acid was reserved for those patients with positive lymph nodes, although surgical and adjuvant treatment was at the discretion of the supervising physician. Prior ethical review of the study was conducted by the Nottingham Local Research and Ethics Committee, who granted approval for the study.

Construction of the array blocks incorporated a wide spectrum of electively resected colorectal tumors and was found to be broadly representative of the colorectal cancer population in the United Kingdom. Two hundred and sixty-six (58%) patients were male and 196 (42%) patients were female. The median age at the time of surgery was 72 y, consistent with a median age at diagnosis of colorectal cancer of 70 to 74 y in the United Kingdom (36). Sixty-nine (15%) tumors arrayed were TNM stage I, 174 (38%) were stage II, 155 (34%) were stage III, and 54 (11%) were stage IV; there were 3 cases of *in situ* disease. These figures are comparable with national figures for distribution of stage I to IV at diagnosis of 11%, 35%, 26%, and 29%, respectively (37). The majority of tumors were adenocarcinomas (392, 85%), and were most frequently of a moderate histologic grade (353, 77%).

At the time of censoring for data analysis, 228 (49%) patients had died from their disease, 64 (14%) were deceased from all other causes, and 169 (37%) were alive. The median 5-y disease-specific survival for the cohort was 58 mo, comparable with a national average of ~45% 5-y survival for colorectal cancer in the United Kingdom (37). The reporting of this study adheres to the REMARK guidelines (38). Investigators conducting statistical analysis were blinded to the clinicopathologic data prior to analysis.

Specimen characteristics. All tumors received following resection in the operating theatre were incised, fixed immediately in 10% neutral-buffered formalin followed by standard processing through to embedding in paraffin wax, ensuring optimal tissue fixation and preservation for histologic examination. The construction of the colorectal tissue microarray has been described previously (33).

Antibody sources. Anti-MIC monoclonal antibody SR99 (a kind gift from Dr. Sophie Caillat-Zucman, Institut National de la Santé et de la Recherche Médicale, Unité 561, Hôpital Saint Vincent de Paul, Paris, France), anti-ULBP1 goat polyclonal antibody (R&D Systems, Inc.), anti-ULBP2 monoclonal antibody M311 (a kind gift from Dr. David Cosman, Amgen, Seattle, WA), anti-ULBP3 goat polyclonal antibody (R&D Systems), anti-RAET1E rabbit polyclonal (this study), anti-RAET1G rabbit polyclonal (39), anti-CD16 mouse monoclonal antibody, and clone DJ130c (AbD Serotec).

Production of polyclonal ULBP4/RAET1E antisera. Recombinant ULBP4/RAET1E protein was produced by cloning the extracellular domain of ULBP4/RAET1E into an NH_2-terminal six-histidine vector (a kind gift from David Owen, Cambridge Institute for Clinical Research, Cambridge University, Cambridge, United Kingdom) and transduction into bacterial pLysis cells. Cultures were induced with 1 mmol/L of isopropyl-L-thio-β-D-galactopyranoside and incubated overnight at 20°C. Inclusion bodies were dissolved in 8 mol/L of urea and purified using nickel beads. Refolding was done by dilution in refolding buffer [100 mmol/L Tris (pH 8), 400 mmol/L arginine, 2 mmol/L EDTA, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione, and 0.1 mmol/L phenylmethylsulfonyl fluoride]. Concentrated protein was run on a 12% SDS-PAGE gel and mass spectrometry confirmed that the band was ULPBP4/RAET1E. The protein was used to immunize rabbits by CovalAB (Lyon, France) to generate serum specific to ULBP4/RAET1E.

Western blot. ULBP/RAET extracellular domains were cloned into pDisplay vector (Invitrogen). Approximately 10^6 Cos7 (a kind gift from Dr. A. Barrow, University of Cambridge, Department of Pathology, Cambridge Institute for Medical Research, Cambridge, United Kingdom) were transiently transfected with 1 μg of plasmid using LipofectAMINE reagent (Invitrogen). After 48 h, cells were harvested into reducing SDS-PAGE buffer and boiled. Lysates were subject to SDS-PAGE and blotted onto Immobilon P membrane. As a loading control, Western blots were also probed with anti–β-actin monoclonal antibody (Sigma-Aldrich) and anti-HA tag monoclonal antibody 6E2 (Cell Signaling Technology) followed by goat anti-mouse HRP (Dako). Blots were also probed with the anti-ULBP/RAET antisera followed by goat anti-rabbit HRP or rabbit anti-goat HRP (Dako).

Flow cytometry. Approximately 10^6 Chinese hamster ovary cells (a kind gift from Dr. A. Barrow) were transiently transfected with 1 μg of the pDisplay plasmids using LipofectAMINE reagent. After 48 h, cells were detached in PBS (1 mmol/L) EDTA and analyzed by flow cytometry. Transfection efficiency was assessed by staining with the anti-HA tag monoclonal antibody followed by goat anti-mouse FITC (Dako). Cells were also stained with the M311 anti-ULBP2 monoclonal antibody and an IgG_{2a} isotype control (Dako). Flow cytometry was carried out on a BD FACSCalibur.

Immunohistochemistry. Immunohistochemical analysis of MIC, ULBP1, ULBP2, ULBP3, RAET1E, and RAET1G expression and $CD16^+$ cell distribution was done using a routine streptavidin-biotin peroxidase method. Tissue array sections were first deparaffinized with xylene and then rehydrated through graded alcohol. To retrieve antigenicity, sections were immersed in 500 ml of citrate buffer (pH 6.0) and heated for 10 min in an 800 W microwave at high power, followed by 10 min at low power. Sections were then immersed in PBS containing 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. To block nonspecific binding of the primary antibody sections were then treated with 100 μL of 1:50 normal blocking serum (Vector Labs) in PBS for 20 min, with the exception of sections to be stained with anti-ULBP1 and anti-ULBP3, which were treated with 100 μL of 1:50 rabbit blocking serum (Vector Labs) in PBS for 20 min. Endogenous avidin/biotin binding was blocked using an avidin/biotin blocking kit (Vector Labs).

Test sections were incubated with 100 μL of monoclonal antibody (SR99) recognizing MICA, with partial cross-reactivity against MICB (40), which was found to show optimal staining at 20 μg/mL in PBS, polyclonal antibody recognizing ULBP1 (Fig. 1A) at 2.5 μg/mL, polyclonal antibody (M311) recognizing ULBP2 (Fig. 1B) at 2.5 μg/mL, polyclonal antibody recognizing ULBP3 (Fig. 1A) at 2.5 μg/mL, polyclonal antibody recognizing RAET1E (Fig. 1A) at 2.5 μg/mL in PBS, polyclonal antibody recognizing RAET1G (39) at 5 μg/mL in PBS, or monoclonal antibody recognizing CD16 at 1:100 dilution in PBS for 60 min at room temperature. Positive control tissue comprised whole sections of colorectal cancer tissue. The primary antibody was omitted from the negative control, which was left incubating in blocking serum.

After washing with PBS, sections were incubated with 100 μL of biotinylated goat anti-mouse/rabbit immunoglobulin (Vector Labs) diluted 1:50 in normal blocking serum/PBS for 30 min, excluding sections incubated with anti-ULBP1 and anti-ULBP2, which were incubated with biotinylated rabbit anti-goat immunoglobulin (Vector Labs) diluted 1:50 in rabbit blocking serum/PBS for 30 min. Sections were washed in PBS and next incubated with 100 μL of streptavidin-biotin/HRP complex (Vector Labs) for 30 min. Subsequently, visualization was achieved using DAB Peroxidase Substrate Kit (Vector Labs). Finally, sections were lightly counterstained with hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted with DPX.

Evaluation of staining. Immunostaining of our TMA sections was evaluated using a ChromaVision Automated Cellular Imaging System with TMA analysis-specific software (ChromaVision Medical Systems). In this system, images of each slide are captured and stored digitally on a computer, and the intensity is calculated automatically, without any reference to clinicopathologic variables. Color threshold settings for the optimal discrimination between brown and blue staining in this system were set prior to analysis and left unchanged throughout. In addition, one investigator who was blinded to the clinicopathology (R.W. McGilvray) reviewed the images of each individual core to confirm both its presence on the slide, and the presence of tumor tissue within the core.

Expression of MIC, RAET1E, and RAET1G was uniform with no negative cores present in the sample. The intensity of staining was used as

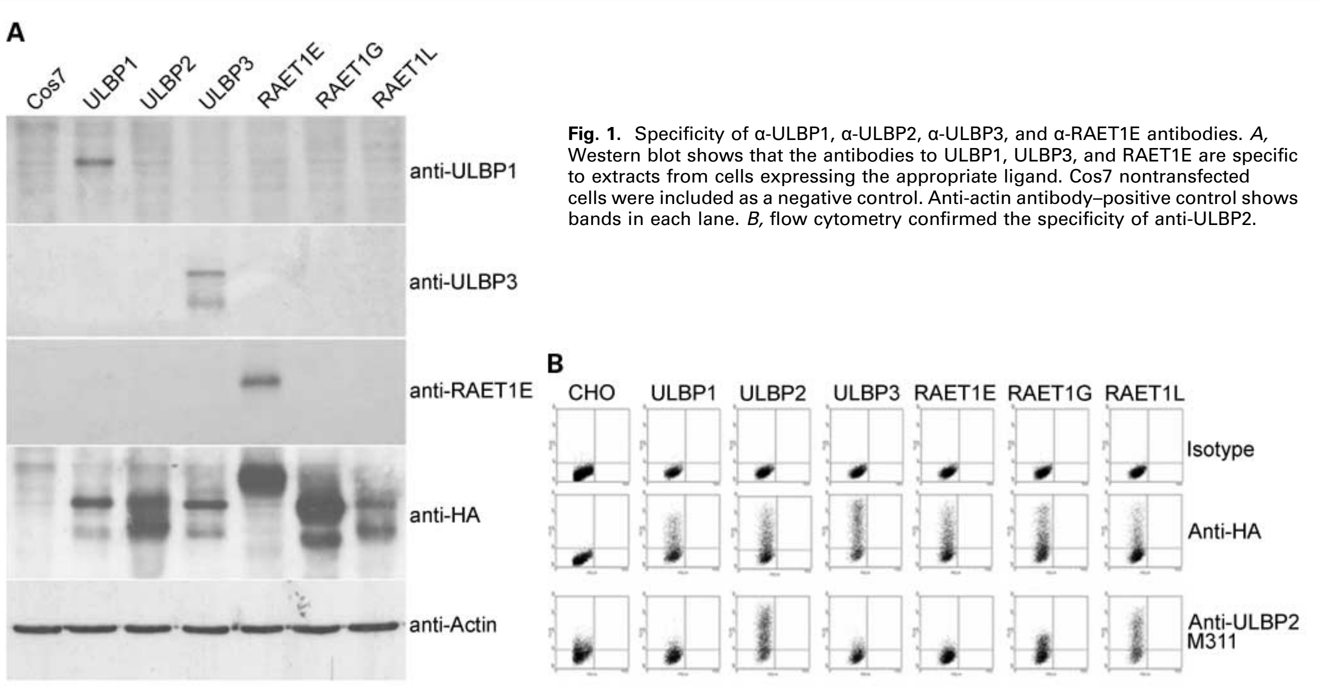

Fig. 1. Specificity of α-ULBP1, α-ULBP2, α-ULBP3, and α-RAET1E antibodies. *A,* Western blot shows that the antibodies to ULBP1, ULBP3, and RAET1E are specific to extracts from cells expressing the appropriate ligand. Cos7 nontransfected cells were included as a negative control. Anti-actin antibody–positive control shows bands in each lane. *B,* flow cytometry confirmed the specificity of anti-ULBP2.

the discriminator with tumors categorized as low and high intensity of expression. This was used as the discriminator for ULBP1, ULBP2, and ULBP3 to maintain the same assessment criteria, although there were a small number of negative cores, which were classed within the low expression group. For analysis of CD16, a cutoff system of positive versus negative was adopted, in which a core was considered positive when any number of positive cells were observed and negative when no cells showed staining. Two hundred and fifty-four of the cores (61%) were completely negative for CD16 cells, with 164 cores positive (39%).

Statistical analysis. Statistical analysis of the study data was done by R.W. McGilvray using the "SPSS" package (SPSS, Inc.), and confirmed by G. Ball using the "Statistica" package (Statsoft, Inc.). Pearson χ^2 tests were used to determine the significance of associations between categorical variables. Disease-specific survival calculations included all patients whose death related to colorectal cancer. Patients whose deaths resulted from non-colorectal cancer–related causes and without evidence of cancer recurrence were censored at the time of death. Kaplan-Meier curves were used to assess factors influencing survival. The statistical significance of differences in disease-specific survival between groups with differing expression was estimated using the log rank test and univariate Cox regression. Significance was established at $P \leq 0.05$. Combined factorial analysis was considered significant at $P \leq 0.025$, applying Bonferroni correction.

Significant prognostic factors were incorporated into a formula that would represent the prognostic outcome for a given individual. This was done by iteratively adding to the variable combination starting at the TNM category. Only factors of independent prognostic significance were retained. Factors having prognostic significance were incorporated into a formula based on the β value derived from the Cox proportional hazard model. This function is not being used to prove or disprove a hypothesis but to show a relationship between a prognostic index and the probability of survival at a given time. As we are not testing significance between populations but showing the correlation between factors, this does not require an assumption of a normal distribution. This approach is largely based on the methods used for breast cancer by Rakha et al. (41). This formula was applied to the study population and the distribution of scores determined. The distribution of scores was then used to derive cutoff values for prognostic groups. These prognostic groups were assessed by plotting Kaplan-Meier curves. From these curves, the 10-y survival percentage was determined. The median prognostic score within each group was then related to survival by production of a scatter plot in Microsoft Excel and fitting an appropriate curve, determined by regression analysis. Where possible, a balance between simplicity and performance was sought to prevent the risk of overfitting.

Results

Comparison of patient/tumor characteristics and prognosis. Relationships between patient/tumor characteristics and disease-specific survival have been published previously (33). We are aware that there may be potential selection bias due to the nature of a single-hospital study, but the distribution of patient sex, age, stage, histologic type, and grade were found to be representative of the colorectal cancer population from the United Kingdom (33). Highly significant relationships were shown between disease-specific survival and TNM stage (log rank, 211.37; $P < 0.001$), and between disease-specific survival and the presence of extramural vascular invasion (log rank, 44.30; $P < 0.001$). There were no other significant correlations found in the analysis.

Expression of individual NKG2D ligands in colorectal cancer and associations with prognosis. A total of six antibodies were used in this analysis to determine the expression of NKG2D ligands on our tissue microarray containing 462 colorectal tumors. Western blot analysis was used to show the specificity of the antibodies to ULBP1, ULBP3, and RAET1E (shown in Fig. 1). The specificity of the anti-ULBP2 reagent was determined by flow cytometry, in which there was good recognition of ULBP2 but an element of cross-reactivity with the highly related molecule RAET1L, and to a lesser extent, with RAET1G (Fig. 1). We are not aware of any antibodies that can specifically

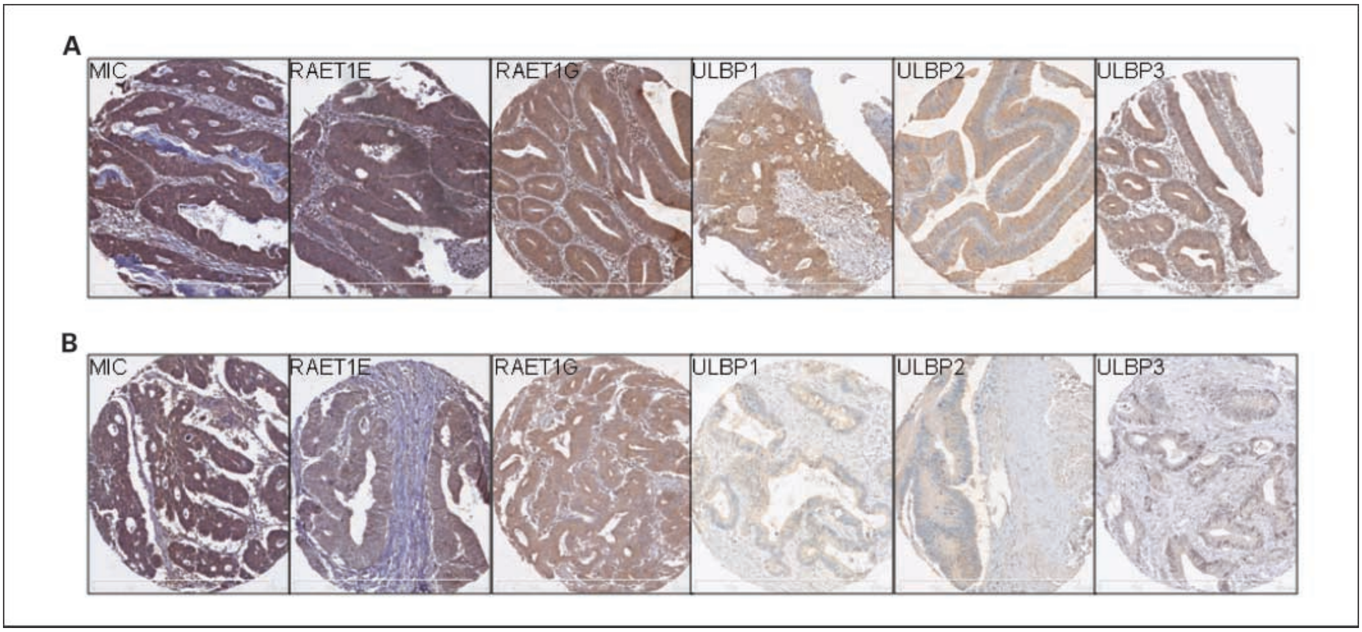

Fig. 2. Typical staining for MIC, RAET1E, RAET1G, ULBP1, ULBP2, and ULBP3. The photographs presented are from representative tissue microarray cores showing high (*A*) and low (*B*) expression at ×100 magnification. *Bar*, 0 to 500 μm in 100-μm increments.

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Table 1. Disease-specific survival analysis of the NKG2D ligands MIC, RAET1E, RAET1G, ULBP1, ULBP2, and ULBP3

Variable	Mean DSS (mo)	Survival range (mo)	Log rank *P*	Cox regression *P*	HR	Lower CI	Upper CI
MICA expression							
High	68	62-74	0.035	0.04	0.76	0.59	0.99
Low	56	47-65					
ULBP1 expression							
High	67	61-73	0.055	0.045	0.81	0.64	1.05
Low	58	50-66					
ULBP2 expression							
High	60	54-66	0.466	0.39	1.13		
Low	67	60-74					
ULBP3 expression							
High	66	58-74	0.53	0.492	0.91		
Low	63	57-70					
RAET1E expression							
High	65	58-72	0.642	0.299	0.84		
Low	64	57-71					
RAET1G expression							
High	74	65-83	0.01	0.03	0.74	0.551	1
Low	62	56-67					
MICA and ULBP1							
Both high	67	60-73	0.018	0.006	0.83	0.7	0.97
Single high	66	57-74					
Both low	50	38-62					
MICA and RAET1E							
Both high	68	62-74	0.02	0.014	0.86	0.77	0.97
Single high	61	51-72					
Both low	44	30-59					
MICA and RAET1G							
Both high	77	68-87	0.003	0.005	0.84	0.74	0.95
Single high	64	56-71					
Both low	54	44-64					
ULBP1 and RAET1E							
Both high	68	59-72	0.041	0.039	0.81		
Single high	62	55-70					
Both low	45	27-63					
ULBP1 and RAET1G							
Both high	73	63-84	0.027	0.032	0.83		
Single high	65	58-72					
Both low	57	48-65					
RAET1E and RAET1G							
Both high	76	67-86	0.004	0.002	0.84	0.77	0.99
Single low/both low	61	55-67					

NOTE: Data presented for each ligand expression case. Combinations of ligand expression are only presented in cases in which statistical significance was found.
Abbreviations: DSS, disease-specific survival; HR, hazard ratio; CI, confidence interval.

discriminate between ULBP2, RAET1L, and RAET1G extracellular domains.[5] All antibodies were reactive against extracellular domains with the exception of the anti-RAET1G antiserum that was raised against the cytoplasmic tail. The specificity of the anti-MIC monoclonal (40) and anti-RAET1G antiserum (39) have been described elsewhere.

The majority of ligands examined in this study were expressed in colorectal cancer with few cores staining negatively. MIC, RAET1E, and RAET1G (Fig. 2) stained in a similar pattern with a broad distribution of intensity from low to high. ULBP1, ULBP2, and ULBP3 (Fig. 2) stained the majority of tissues but showed a skewed distribution in which the staining of tumor tissue was mostly at the lower scale of intensity. In each of the six cases, the median intensity of staining was used to separate the two populations corresponding to high and low expression. There was no visible staining of nucleus or stromal elements in any of the analysis carried out.

When analyzed individually for effect on survival (summarized in Table 1), MIC and RAET1G expression showed significant survival advantages. Kaplan-Meier survival analysis of MIC (log rank $P = 0.035$, Cox regression $P = 0.018$) and RAET1G (log rank $P = 0.01$, Cox regression $P = 0.03$; Fig. 3) showed a significant association between disease-specific survival and high levels of expression. The mean survival at 10 years for MIC expression was 56 months in the low expression group, compared with 68 months for the group with high expression levels. For RAET1G expression, the mean survival at 10 years was 62 months in the low expression group and

[5] R.A. Eagle, unpublished data.

74 months in the high-expressing patients. Median survival for high expressing patients could not be calculated in either case as >50% of these patients were still alive at the data end point.

The ligands RAET1E and the ULBPs did not show any statistically significant association with disease-specific survival. When considered in univariate analysis against the clinicopathologic factors, the expression of MIC correlated with tumor grade ($P = 0.037$), RAET1E with TNM stage ($P = 0.017$), ULBP1 with tumor grade ($P = 0.006$), and ULBP3 with Duke's stage ($P = 0.022$). There was no association with MSI which does not confer a survival benefit in this cohort of patients.

NKG2D ligand expression is heterogeneous and combinations of ligands are highly associated with prognosis. Ligands for NKG2D are not expressed independently on the cell surface (2) and we were interested to find out what the prognostic effect of combined analysis would be. Each ligand was combined with each of the five other ligands and then subjected to statistical analysis as discussed previously. Although it would be of major interest to analyze the expression of more than two ligands at any one time, this is not feasible due to statistical limitations. The statistical power of the cohort is lost with the large number of groups generated. The significance of each ligand expression in multifactorial analysis, tested by the Cox regression model, is discussed later.

MIC expression considered on its own had a P value of 0.035, so when MICA and RAET1G expression patterns were combined, the relationship between both factors and disease-specific survival was shown to be highly significant ($P = 0.003$; Fig. 3). Certain ligands that on their own had no effect on significance were found to have an association with survival when considered in combination with another ligand. RAET1E had no significant association with survival unless combined with RAET1G expression, which indicates an improved prognosis ($P = 0.004$; Fig. 3). In the same manner, the combination between RAET1E and MIC expression produced a P value of 0.02 (Fig. 3) in relation to disease-specific survival. This seems to indicate a close association between ligands that is not directly related to patient survival. The combination of ULBP1 and MIC gave a P value of 0.018 (Fig. 3), indicating a similar relationship. These data provide an indication that NKG2D ligands are cooperative in a manner that could, either directly or indirectly, affect patient survival.

All combinations were analyzed but no combinations other than those discussed here showed any statistically significant association with disease-specific survival.

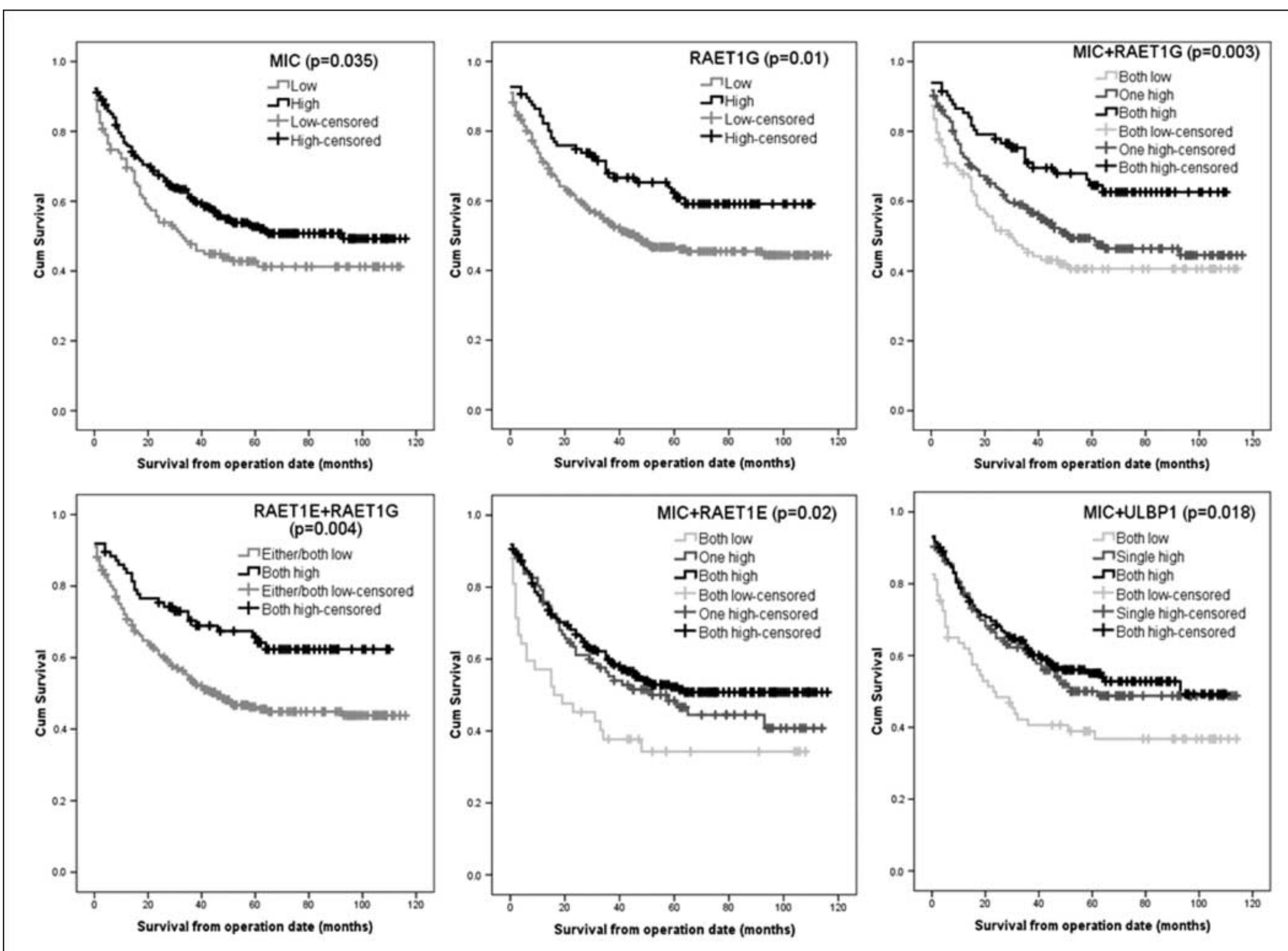

Fig. 3. Kaplan-Meier plots showing disease-specific survival in statistically significant cases.

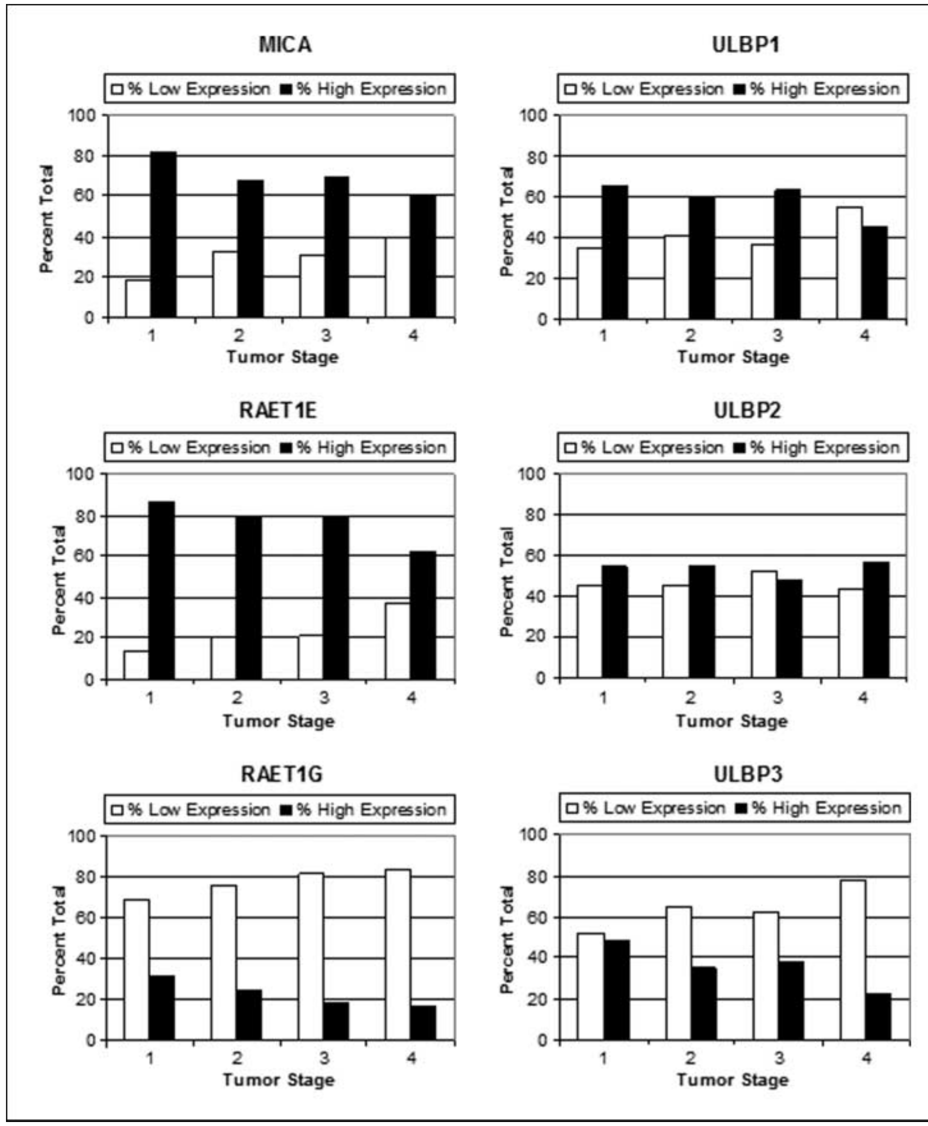

Fig. 4. Tumor expression of NKG2D ligands decreases as tumor grade increases. *Columns*, number of patients classified as low or high expression represented as a percentage of the total for each TNM stage in which stages 0 and I were classified together as 1. Stages II, III, and IV were left as individual groups.

High-level NKG2D ligand expression reduces with increasing tumor stage. The patient data for expression of each ligand was divided according to their TNM classification (as 1, 2, 3, and 4), where 1 included patients with stage 0 and I tumors. Each stage was subdivided into high and low expression as a percentage of the total (as shown in Fig. 4). When considering all the expression data, it was noted that a trend occurred in all but one case. As tumor stage progresses, the frequency of high-level expression decreases through to stage IV, which has the worst prognosis. This data trend shows a direct relationship between the patients' worsening prognosis and loss of expression, indicating that late stage tumors have been driven towards lower expression levels.

Multivariate analysis uncovers potential relationships between NKG2D ligands in overall survival of patients with colorectal cancer. Multivariate analysis highlighted the factors with a link between the clinicopathologic data and the ligands tested were TNM stage ($P < 0.001$) and MIC expression ($P = 0.012$). Although Cox regression analysis of RAET1G ($P = 0.029$) showed significance, it seems to be co-correlated with TNM stage as this significance was lost in multifactorial Cox regression analysis ($P = 0.244$). Other combinations did not show independent prognostic value. This is likely to be due to the partial co-correlation and interdependence of parameters.

β values were used to derive a prognostic scoring formula for the two prognostic factors. TNM stage had a β value of 0.717 and MIC had a β value of -0.381. This resulted in the following formula:

$$\text{Prognostic Score} = (0.717 \times \text{TNM stage}) - (0.381 \times \text{MIC status})$$

Prognostic scores were calculated for the experimental population and the distribution of scores determined (results not shown). Prognostic categories were defined based on this distribution. Kaplan-Meier survival curves were plotted for these categories and the median 10-year survival determined for each category. Regression analysis was used to define the relationship between median category and percentage of 10-year survival. A good relationship was seen between score and

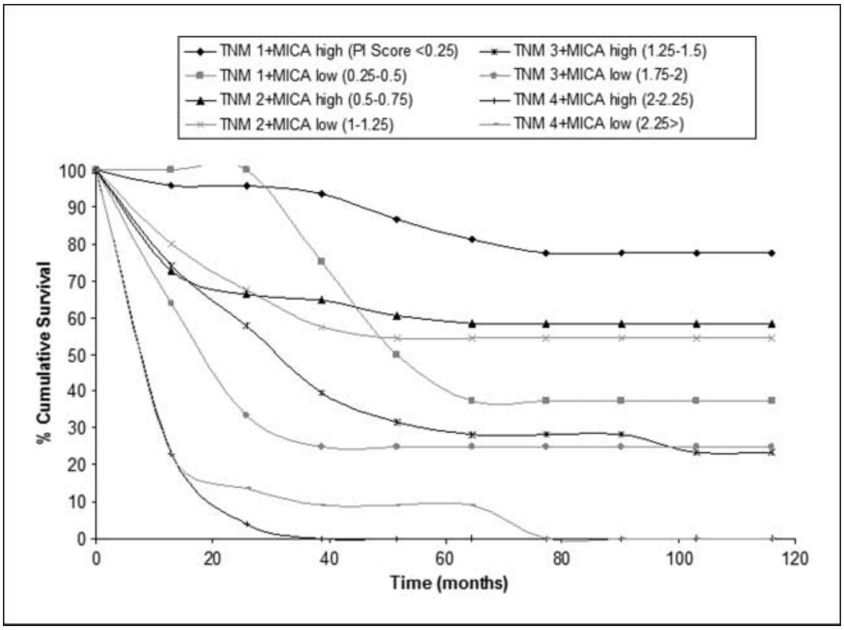

Fig. 5. Ten-year survival plot of patient prognostic groups according to their prognostic index score. *Points*, cumulative survival for each of the prognostic groups as subdivided according to our prognostic model generated from the Cox regression analysis. The prognostic model was generated based on TNM category and MIC expression level. Each prognostic group is indicated in the legend (*top*) with the corresponding prognostic index score.

median 10-year survival having an r^2 = 0.912. This initial prototype prognostic index has been developed on a single center sample set. As such, it should be validated in other centers for prognostic performance.

The cumulative survival plot for patients sorted according to their prognostic index score (Fig. 5) identified unusually poor survival in group 2 patients. This group included patients who were classified as TNM stage I, with low MIC expression levels, and according to the prognostic model, have a median 10-year survival rate of 50%. When we consider group 1 patients who have the same TNM classification but high MIC expression, their median 10-year survival was 83.8%. The expected survival rate of TNM stage I colorectal cancer patients is 80% to 95%, so those patients with low levels of MIC expression should be selected for adjuvant therapy following surgical resection of their tumor.

As MIC was the only ligand shown to be an independent marker of good prognosis in colorectal cancer, its expression was correlated with innate cell infiltration. CD16 is expressed by innate mononuclear cells including NK cells and monocytes, which both express high levels of NKG2D. There was a strong positive correlation (P = 0.004) between MIC expression and cellular infiltrate.

Discussion

After remaining an unproven theory for many years, cancer immunosurveillance has undergone a renaissance (21). Diverse lines of evidence from *in vivo* models suggest that the immune system attacks early stage tumors. Cancer cells that survive must adapt to avoid the immune system, a process variously described as immunoediting, immune sculpting, or cancer immune evasion (21). Studies with *in vivo* cancer models strongly suggest that the activating immune receptor NKG2D is involved in anticancer immune responses (18–20, 29, 42). In humans, both primary tumors and tumor-derived cell lines frequently express NKG2D ligands (11, 12, 14). In this study, we show that there is a good correlation between MICA expression and infiltration of colorectal tumors with innate mononuclear cells.

Tissue microarray technology allows protein expression to be assessed and compared in a large number of samples simultaneously. This allows the identification of broad trends in expression patterns, and correlations to be made between expression, clinicopathologic features, and patient prognosis. We have previously shown that MICA has independent prognostic value in colorectal carcinoma using polyclonal antisera (33). In this study, we confirmed this result with a monoclonal antibody that recognizes MICA and MICB, showing that high expression is associated with improved patient survival. The study was extended using antibodies that recognize all members of the ULBP/RAET family of NKG2D ligands. The first observation from this analysis was that NKG2D ligand expression was heterogeneous in primary cancers, as not all ligands were expressed highly in the same tumor. Individually, MIC and RAET1G showed an association with prognosis, but other NKG2D ligands did not. Significantly, the combinations of these two ligands were as good as TNM stage at predicting patient prognosis. These results make a strong case for NKG2D-mediated immunity involvement in human colorectal cancer development.

There are two explanations for NKG2D ligand heterogeneity on tumors that are not mutually exclusive. First, the heterogeneity might reflect the fact that NKG2D ligands have different promoters and can be expressed independently in response to different stress response pathways. Also, there is now evidence for posttranscriptional regulation of NKG2D ligand expression which might also allow differential regulation of the expression of different NKG2D ligands, an example being the potential role of micro-RNAs (43). Some stimuli have been reported to result in the expression of all NKG2D ligands tested, such as DNA damage responses in mice (15). Others have been shown to be specific to some NKG2D ligands; for example, BCR/ABL regulates MICA but not ULBP1-2 in K562 cells, and treatment of hepatoma cells with histone deacetylase inhibitors induced MICA, but not ULBP1-3 (16, 44).

If this explanation was exclusively true, it would imply that heterogeneous NKG2D ligand expression on tumors was simply reflecting the activation stage of various cancer-related pathways, and was not a result of selective pressure placed on the cancer by the immune system to develop evasion strategies. In perforin knockout mice that are deficient in immune cell cytotoxicity, chemically induced tumors are seen to have much higher expression of Rae-1 than wild-type mice. In NKG2D knockout mice, early arising prostate tumors have higher levels of NKG2D ligand expression than tumors arising in wild-type mice. This indicates that cytotoxic immune responses, and NKG2D mediated immunity, can place selection on developing tumors to switch off NKG2D ligand expression as part of a cancer immune evasion strategy or immunoediting. Our study indicated that for all six NKG2D ligands, expression was highest in the early stage I tumors, but then decreased in later stages II, III, and IV, with highly aggressive stage IV tumors having the lowest expression. The data are consistent with a model in which developing cancers lose the expression of NKG2D ligands to avoid the immune system. This does not preclude other NKG2D-specific cancer immune evasion mechanisms being important, such as the release of soluble NKG2D ligands or immunosuppressive cytokines such as transforming growth factor-β (25–27).

It has been suggested that NKG2D ligands are important in the immune response against tumors and we have shown this both through our prognostic model and the significant correlation between MIC expression and NK cellular infiltration. The poor prognosis of early stage colorectal cancer patients with low MIC expression is evident and has not previously been described. Currently, the generally accepted regimen for TNM stage I patients is surgery alone, although our data suggests that the group of patients with low-level MIC would benefit from additional adjuvant therapy. Patients having colonoscopy could easily have a biopsy sample histologically stained for NKG2D ligand expression to further classify their tumor in line with our results.

In conclusion, tissue microarrays provide a further line of evidence for NKG2D involvement in cancer immunosurveillance and combinatorial analysis of NKG2D ligand expression is an attractive target for the development of improved prognostic classification of colorectal and other carcinomas.

Disclosure of Potential Conflicts of Interest

R.A. Eagle and J. Trowsdale hold a patent on ULBP5/RAEIG. The other authors disclosed no potential conflicts of interest.

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Roger W. McGilvray, Robert A. Eagle, Nicholas F.S. Watson, et al.

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