Expression of MHC Class I Polypeptide-Related Sequence A (MICA) in Colorectal Cancer

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Research Article

Keywords: MICA, IHC, colorectal cancer, expression, prognostic marker

Posted Date: March 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-311547/v1

Abstract

Background: The major histocompatibility complex class I polypeptide-related sequence A gene (MICA) is one of the ligands of NKG2D activating receptor. MICA stimulates NKG2D that further triggers activation of natural killer cells which leads to killing of infected target cells. Tumor cells utilize escape strategies to subvert the biological function of NKG2D by shedding overexpressing MICA. In this study, we determine the levels of MICA colorectal cancers (CRCs). Additionally, we establish correlations between MICA expression and clinical characteristics. Publicly available data and bioinformatics tools are used for validation purposes.

Methods: We determined the MICA RNA expression levels and correlation with clinicopathological parameters in CRC using UALCAN web-portal. We performed immunohistochemical analysis on tissue microarrays having 192 samples, acquired from 96 CRC patients to validate the expression of MICA in CRC and adjacent uninvolved tissue and investigated its prognostic significance by Kaplan-Meir and proportional hazards methods.

Results: Bioinformatics and immunohistochemical analyses showed that MICA expression was significantly upregulated in CRCs as compared to uninvolved and the overexpression of MICA was independent of pathologic stage, histotype, nodal metastasis status, p53-status, as well as patient's race, age and gender. Moreover, PROGgeneV2 survival analysis of two cohorts showed poor prognosis of CRC patients exhibiting high MICA expression.

Conclusions: Overall, our findings demonstrate high expression of MICA, suggest poor prognosis of CRC patients exhibiting high MICA expression. These results can be further explored due to its potential to provide clues to the mechanistic contributing role of the tumor microenvironment to the progression of progression of CRC.

Background

As a leading cause of cancer-related deaths in Americans, cancer of colon and rectum remains in third position for new estimated cases and mortality according to American Cancer Society in 2020 [1]. Current diagnosis of CRC is based on tumor-node-metastasis (TNM) stage, which lacks the interpretation of epigenetic background and genetic variants. Therefore, identification of new biologic markers is a promising approach to improve detection of aggressive phenotypes and provide better guidelines for clinicians towards CRC treatment.

MHC class I polypeptide-related sequence A (MICA) is a cell surface protein overexpressed under stress conditions [2]. Upon interaction with natural killer group 2D (NKG2D) receptors, MICA promotes activation of natural killer (NK) cells, and IFN-gamma secretion and degranulation of perforin and granzymes, leading to apoptosis of cells expressing high levels of MICA [3, 4]. Due to its functions, MICA works as an important component of the immunosurveillance system to combat infections by foreign pathogens and conditions that lead to cellular stress, including cancer [5]. Studies show that MICA has a soluble isoform

(sMICA), which is reported to be highly expressed in aggressive forms of cancer and to reduce the cytotoxic activity of NK cells [6]. Therefore, MICA has been proposed as a relevant player of the tumor microenvironment (TME) [7], worth to be explored as a factor of tumorigenesis.

Aberrant expression of MICA has been described in different types of cancers, including prostate, lung, stomach, and cholangiocarcinoma [8]. Despite the level of information, the role of MICA expression in tumorigenesis is not totally clear. In carcinoma of the prostate [9], gastric cancer [10], and non-small cell lung cancer [11], higher expression of MICA related to better prognosis. On the other hand, higher expression in patients with pancreatic cancer [12], breast cancer [13], hepatocellular carcinoma [14], and non-small cell lung cancer [15] predicted for poor outcome. In relation to CRC, elevated expression of MICA has been found in tumor tissue as compared to normal specimens [16]. However, it was reported improved disease-specific survival for patients with high expression of MICA [17, 18].

Since data suggest MICA as a molecule of the TME with an emergent role as a marker of aggressive disease, further investigations are needed to establish its prognostic value in CRC. Herein, we determine the tumor levels of MICA in patients with CRC. Additionally, we establish correlations between MICA expression and clinical characteristics. Publicly available gene expression data and bioinformatics tools are used for validation purposes. Our findings agree with published literature for higher expression of MICA in CRCs, however, contrary to prior reports in CRC [17, 18], they point out to poor prognosis for patients whose CRCs exhibit high MICA expression. In all, our findings suggest additional work is needed to establish the role of MICA expression as a discriminator of aggressive CRC.

Methods

Bioinformatics analysis: The UALCAN platform was used to access MICA mRNA levels in normal (uninvolved) colon and CRC tissues [19]. This resource for gene expression analysis uses data from The Cancer Genome Atlas (TCGA). mRNA data are expressed as transcripts per million and representative of standard deviations from the median across samples for the given cancer type. PROGgeneV2, prognostic database [20], was used to perform Kaplan Meier and proportional hazards survival analyses in CRC patients associated to mRNA levels of MICA (GSE41258 and GSE29621 independent publicly available data sets).

Patients and tissue samples: The study population was derived from the University of Mississippi Medical Center (UMMC), Jackson, MS, USA. Specimens collected (2006-2016) following surgery were de-identified and later provided a unique study identification. Clinical and pathological characteristics of study subjects are provided in **Table 1**.

Table 1: Clinicopathological characteristics of patients	
Characteristic	**Finding**
Age, years, mean (range)	59.2 (23-87)
Sex, Number (%)	
Male	50 (52.1%)
Female	46 (47.9%)
Race/ethnicity, number (%)	
African Americans	56 (58.3%)
Non-Hispanic Whites	40 (41.7%)
Site, Number	
Colon	62 (64.6%)
Rectum	34 (35.4%)
TNM stage, number (%)	
I	11 (11.4%)
II	30 (31.3%)
III	35 (36.5%)
IV	20 (20.8%)
Histological grade, number (%)	
Well-differentiated	6 (6.3%)
Moderately differentiated	78 (81.3%)
Poorly differentiated	7 (7.3%)
Unknown	5 (5.1%)
Lymph node metastasis, number (%)	
Negative	36 (37.5%)
Positive	50 (52.1%)
Unknown	10 (10.4%)
Surgical margins, number (%)	

Negative	74 (77.1%)
Positive	18 (18.8%)
Unknown	4 (4.1%)
Follow-up time (years), median (range)	4.6 (0.1-10.3)

The data includes sex, race, TNM stage, histological grade, evidence of LNM, surgical margins, survival times, and status. Tumor and normal colonic tissues, adjacent to tumor, were obtained immediately after operation. We included 96 cases, assessed by a board-certified pathologist (VS). Staging was performed according to the guidelines of the American Joint Committee on Cancer. Following surgery, clinical follow-up data were obtained, with the median follow-up of 5.4 years (range 0.1-10.3 years) for the 96 patients. Due to its retrospective nature, the Institutional Review Board waived the need for informed consent. The study was approved by the University of Mississippi Medical Center (UMMC) Institutional Review Board under protocol # 2012-0205. All methods were performed according to standards set by the Declaration of Helsinki.

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Construction of tissue microarrays: Tumor stage-matched tissues were used to create tissue microarrays (TMA). For each patient, representative formalin-fixed paraffin-embedded (FFPE) tissue blocks including a normal block and a tumor block. A total of 192 samples for the TMA construction were included in the final composite block. Based on the verified histological features, FFPE blocks of primary tumors were selected by the pathologist. From the primary FFPE blocks, cylindrical cores of 2-mm diameter were transferred to paraffin blocks using a Beecher MTA1 Manual Tissue Arrayer (Beecher Instruments, Sun Prairie, WI). For IHC staining and analysis, the resulting TMA composite blocks were sectioned at 5-μm thickness.

***Immunohistochemistry*:** As described before [9, 21], IHC was performed according to manufacturer's instructions provided in ABC Kit (Vector Laboratories Inc., Burlingame, CA). Following antigen retrieval, with a citrate buffer (pH 6.0) for 20 min, and incubation with 3% hydrogen peroxide, the FFPE TMA sections were deparaffinized and rehydrated during 10 min. To block unspecific binding, the slides were treated with Protein Block Serum-Free (Cat X0909, Dako, Santa Clara, CA) for 12 min followed by incubation with 10% normal serum for 1 h at room temperature. Next, the TMA slides were incubated with rabbit anti-human primary polyclonal antibody against MICA in 1:25 dilution (Cat# PA5-35346, Thermo Scientific, Waltham, MA) overnight at 4°C. Next, the slides were washed with phosphate-buffered saline (PBS), incubated with components of the ABC kit, and with 3, 3-diaminobenzidine (DAB) for color development. Slides were counterstained in hematoxylin and mounted. Subcellular localizations of MICA were defined as cytoplasmic/membranous or globular staining by the pathologists, and scored. Evaluation of IHC was independently performed by two independent evaluators blinded to the specific diagnosis or prognosis for each individual case. To assess the MICA cytoplasmic staining intensity, a

modified version of the “quickscore” method was utilized [9]. Data are expressed as median (interquartile range). To assess the association between MICA expression and clinical features in the CRC cases, patients were dichotomized by low and high MICA tumor expression, based on the optimal cutoff point calculated as the value with the most significant log-rank test split (3.4 for combined intensity score).

Statistical analysis: The SPSS software package, version 13.0 (SPSS Inc., Chicago, IL USA), SAS 9.4 (SAS Inc., Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA were used to analyze the data. The difference in MICA gene expression between uninvolved tissue and tumor tissue or for any other pairwise comparison obtained using bioinformatics analyzes was evaluated by Student's t-test. One-way ANOVA and Dunnett's multiple comparisons were utilized when three or more groups were compared. Pairwise comparisons were always relative to normal tissue. For IHC data, differences were compared by Mann-Whitney U test for non-matched data or Wilcoxon signed rank test for matched-pairs. Two-sided P-values were determined via Chi-square or Fisher's exact tests for categorical variables. Overall survival was analyzed by the Kaplan-Meier and proportional hazards methods with the use of the log-rank test and hazard risks (HR) and 95% confidence intervals (95% CI) to compare overall survival. For all analyses, the level of significance was set at $P<0.05$.

Results

Bioinformatics analyses of RNA expression of MICA in CRC tissues: Inspection of uninvolved tissues (N=41) vs. primary CRC (N=286) **(Figure 1A)** by UALCAN database revealed a 30% increase of expression of MICA mRNA in CRC relative to normal epithelium (P=1.794E-07). Furthermore, we found elevated expression of MICA in CRCs of patients for both sexes [males (n=41) and females (n=156), $P<0.0001$ for each comparison] (**Figure 1B**). Further analysis of MICA transcripts based on individual cancer stages [stage 1 (n=45), stage 2 (n=110), stage 3 (n=80), and stage 4 (n=39)] revealed high expression for all cancer stages, relative to normal tissue ($P<0.0001$) for each comparison (**Figure 1C**), however no differences were observed between individual stages. Next, we determined the association of MICA mRNA with patient’s race. MICA transcripts were elevated regardless of race in CRC, when tumors of Caucasian (n=193), $P<0.0001$; African-American (n=55), P=0.002; and Asian (n=11), P=0.022 CRC patients were compared to uninvolved tissue (**Figure 1D**). When analyzed by age (**Figure 1E**), MICA mRNA was not different between normal tissue and tumor obtained from 12 individuals aged 21-40 years old (P=0.06). MICA transcripts, however were significantly elevated for all older groups [41-60 years (n=90), $P<0.0001$; 61-80 years (n=149), $P<0.0001$; and 81-100 years (n=32), P=0.002], relative to normal epithelium. Further analysis showed MICA RNA expression was higher in CRCs based on histological subtypes than uninvolved tissues (**Figure 1F**). Expression was high, both for adenocarcinoma (n=243), $P<0.0001$ and for mucinous adenocarcinoma (n=37), $P<0.0001$ relative to normal tissue. However, no differences in transcript levels were noted between adenocarcinomas and mucinous tumors. In addition, MICA expressions in three distinct nodal metastasis status [N0 (n=166), N1 (n=70), and N2 (n=47); $P<0.0001$ for each comparison] were all upregulated, but comparable, as compared to non-tumorous tissue (**Figure 1G**). Likewise, MICA expression based on p53-status was elevated in CRCs. It was found that 160 CRC patients with p53-wild type and 122 patients with p53-mutated status exhibited higher

MICA expression (**Figure 1H**), P<0.0001 for each case. Transcripts of MICA, however were not different between tumors from patients with p53-wild type or p53-mutate status.

Association between expression of MICA transcripts and survival of CRC patients: Using the prognostic database PROGgeneV2, we retrieved and performed survival analyses on the datasets GSE41258 and GS29621, using the median value as threshold. In both datasets, significantly poorer prognosis was found for patients with high MICA mRNA levels relative to those with low MICA mRNA (log rank, P=0.014, HR: 2.15, 95% CI: 1.17-3.94 for GSE41258 and log rank, P=0.003, HR: 9.87, 95% CI: 2.18-44.69 for GS29621) (**Figures 2a and 2b**).

MICA protein expression by immunohistochemical (IHC) profiling of normal colonic and tumor tissues: Out of 384 cores, a total of 74 cores were unsuitable and excluded from analysis due to loss of tissue or lack of viable cells within the core. Higher MICA expression was observed as globular/nuclear or cytoplasmic in cells from normal tissues **(Figure 3A)**. Nuclear staining was observed in 11.6% (8 of 69) positively stained uninvolved cores. Cytoplasmic immunostaining was observed in 40.6% (28 of 69) normal cores. In both the basal and luminal portions of colonic crypts, staining was observed mainly in the cytoplasm of epithelial cells and the peripheral cytoplasm of Goblet cells, with negative reactivity to mucous glands. In CRCs, MICA staining was predominantly cytoplasmic, as noted in 84.9% (73 of 86) of the positively stained cores **(Figure 3A)**. Globular staining was present in 32.6% (28 of 86) of positively stained specimens **(Table 2)**. High expression was also observed in mucinous tumors as well as moderately and poorly differentiated adenocarcinoma (**Figure 3B and 3C**). Analysis of nuclear immunostaining revealed a 3.5-fold higher combined intensity score in CRCs (1.4 ± 2.5) relative to normal glandular samples (0.4 ± 1.4), P=0.002. Likewise, cytoplasmic immunostaining was 2.4-fold higher when the combined intensity score in CRCs (3.4 ± 2.8) was compared to normal glandular samples (1.4 ± 2.1), P<0.0001 **(Figure 3D and Table 2)**. Due to higher prevalence of cytoplasmic immunostaining in CRCs, we used this value to perform further analyses.

Correlation between MICA tumor expression, clinical pathological features and patient survival in UMMC cohort: In order to assess the association between MICA expression and CRC clinical features, UMMC patients were divided into low and high MICA tumor expression based on the optimal cutoff point calculated based on the median (3.4) of cytoplasmic staining. Correlations between the two groups and clinical features were calculated using Fisher's exact test (**Table 3**). There was not significant association of MICA expression with patients' sex (P=0.277), race/ethnicity (P=0.665), age (P=0.821), tumor site (P>0.999), surgical margins (P=0.404), LNM (P>0.999), N stage (P>0.999) or clinical stage (P=0.817). However, high MICA tumor immunoreactivity was associated with higher T stage (P=0.020). There was correlation between high MICA expression and poor OS, however the association was not statistically significant (log rank, P=0.213; HR: 1.206, 95% CI: 0.6947-2.115) **(Supplementary Figure 1)**, nor following stratification by race/ethnicity, sex, age, site, surgical margins, or tumor stage (data not shown).

Table 2. Frequency of MICA expression in colonic tissue according to tissue type and localization.

Tissue/Localization	Frequency	MICA average combining stage score
Normal/Cytoplasmic	28/69 (40.6%)	1.4±2.1*#
Tumor/Cytoplasmic	73/86 (84.9%)	3.4±2.8*#
Normal/Nuclear	8/69 (11.6%)	0.4±1.4*#
Tumor/Nuclear	27/86 (31.4%)	1.4±2.5*#

*P<0.05 when comparing between different tissue types in the same cellular sub localization. #P<0.05 when comparing between different cellular sub localization in the same tissue type.

Table 3. Correlation of clinicopathologic findings with cytoplasmic MICA expression			
	MICA low	**MICA high**	***p* value**
Sex			
Female	16	27	0.277
Male	22	21	
Race			
African American	24	28	0.665
Caucasian American	14	20	
Age			
< 55 years	13	18	0.821
≥ 55 years	25	30	
Site			
Colon	27	33	>0.999
Rectum	11	15	
Surgical margins			
Negative	31	36	0.404
Positive	5	10	
Lymph node metastasis			
Negative	14	16	>0.999
Positive	22	25	
T stage			
1-2	11	4	0.020*
3-4	27	44	
N stage			
0	13	17	>0.999
1-2	24	29	
Clinical stage			
I-II	12	17	0.817
III-IV	25	29	

*P<0.05.

Discussion

CRC mortality rate is elevated worldwide. Even though the five-year survival of CRC patients has improved due to early detection, close to 25% of patients still are diagnosed with stage 4 disease. As the relative 5-year survival rate of patients with metastatic CRC (mCRC) remains poor [22], there is an urgent unmet need to develop a more effective treatment for patients suffering from this disease. PD1 inhibitors have been a successful immunotherapy approach to a specific subgroup of mCRC, that are mismatch-repair-deficient and microsatellite instability-high [23]. Ongoing research is focused on looking for treatments for other subgroups of mCRC. Emerging approaches include targeting of the TME, which might complement immune checkpoint inhibition. To this end, in the current study we evaluated MICA as a potential TME marker for aggressive disease. Analysis by UALCAN database in CRC suggested that MICA expression was closely associated with individual cancer stages. In addition, MICA expression in UMMC CRC cohort, assessed by IHC, was increased in CRCs and was associated to features of aggressive disease.

Expressed in different malignancies, MICA is considered an important component of the tumor immunosurveillance by interacting with the receptor NKG2D and activating NK cells and co-stimulating subtypes of T-cells [24-26]. Our results showed increased expression of MICA in CRC compared to uninvolved tissue. Interestingly, higher MICA expression was significantly associated to increased tumor stage (T3 and T4), suggesting the potential of MICA as a marker for aggressive CRC.

There is disagreement in the literature about the association between MICA expression and the prognosis of cancer patients. Increased tumor levels of MICA were previously associated to good prognosis in prostate cancer and cervical cancer [27]. However, elevated MICA was reported as an indicator of poor prognosis pancreatic cancer [12] and breast cancer [13]. Survival analysis performed in our cohort suggests a possible association of poor prognosis to higher expression of MICA in CRCs (Supplementary Figure 1), supported by the PROGgeneV2 survival analysis in two distinct cohorts (Figure 2). In disagreement with our findings, two independent studies indicated better prognosis in patients with expression of MICA in CRC [17, 18]. Because this controversy has been also found in other tumor sites such as Non-Small Cell Lung Cancer [15, 28] and gastric cancer [10, 29], the matter of expression of MICA and its association with outcome remains an issue of active debate.

In different tumors, MICA sheds from the cell surface into the circulation as soluble component (sMICA). Binding of sMICA to NKG2D receptor, without activation or co-stimulation of the effector cells, and promotes tumor escape. Unfortunately, we did not have access to plasma to assess circulating levels of sMICA or NKG2D levels in NK cells. This would help us understand our results on scope of the described tumor immunoevasion strategy mediated by MICA. In aggressive pancreatic carcinoma, an inverse correlation was found between expression levels of soluble MICA and NKG2D [12]. Moreover, findings of a recent study concluded that high MICA serum levels are associated with poor prognosis of

hepatocellular carcinoma (HCC) [30]. Findings from this study also suggested that MICA blocks NKG2D signaling pathway by mediating tumor-immune escape in HCC [30]. This pathway would further protect tumor cells from NK cell-mediated cytotoxicity in CRC. Further studies are needed to evaluate protein expression of NKG2D expression in CRC, its association with MICA, and mechanistic basis of the interaction between these two molecules. Benefits include development of innovative TME-based immunotherapy strategies.

Immune checkpoint blockade therapy has achieved limited success within CRC patients. Current research has been focusing on combined treatment with immunotherapy to improve the outcome of patients with aggressive form of the disease, including chemoimmunotherapy, immunotherapy with radiation therapy and others. A potential option is the stimulation of NK cells and cytotoxic T cells through stimulation of MICA expression and neutralization of sMICA. However, additional research is needed to clarify the divergent information related to the expression of MICA in CRC tumors as well as its prognostic value, and mechanistic involvement in disease aggressiveness.

Conclusions

Our study provides evidence to up-regulation of MICA in CRC and suggests poor prognosis of CRC patients exhibiting high MICA expression. We believe that relevance of our findings is high due to similar patterns of high MICA expression identified in large, publicly available omics databases, and the potential of MICA as an actionable molecule of the TME.

Abbreviations

CRC Colorectal cancer

DFS Disease Free Survival

FFPE Formalin-Fixed Paraffin-Embedded

GI Gastrointestinal

IHC Immunohistochemistry

LNM Lymph Node Metastasis

MICA MHC class I polypeptide-related sequence A

TMA Tissue microarray

TNM Tumor-Node-Metastasis

UMMC University of Mississippi Medical Center

Declarations

Ethical approval

The study was approved by the UMMC Institutional Review Board.

Ethics approval and consent to participate: Institutional Review Board waived the need for informed consent given the retrospective nature of the study. The study was approved by the University of Mississippi Medical Center (UMMC) Institutional Review Board under protocol # 2012-0205. All methods were performed according to standards set by the Declaration of Helsinki.

Consent for publication: Not applicable

Availability of data and materials: Datasets utilized for bioinformatics analysis are publicly available (GSE41258: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41258; GSE29621: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29621). Histopathology data are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: This study was supported by the Office of Research and Sponsored Programs, University of Mississippi Medical Center (IE and CRG); Coordination for the Improvement of the Higher Education Personnel (CAPES) Foundation, Scholarship #13603-13-2 (MJS), and the Impact Funds from School of Medicine and the Department of Pathology, University of Alabama at Birmingham (UM).

Authors' contributions: IE, SA, MS, VS, WSO, and SAD contributed to the conceptualization of the research concept, performing the experiments, formal analyses, and writing the original draft. AP, UM, and CRG contributed to the conceptualization of the research concept, study design, supervision, reviewing and editing the original draft, and funding acquisition.

Acknowledgements: We thank Elizabeth Tarsi, Tara Craft, Eldrin Bhanat, Jaswinder Kaur, and Joy King for establishing and maintaining the databases. We thank Dr. Amit Reddy at the University of Mississippi Medical Center for technical assistance and Dr. Donald Hill at the University of Alabama at Birmingham for editing the manuscript

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Figures

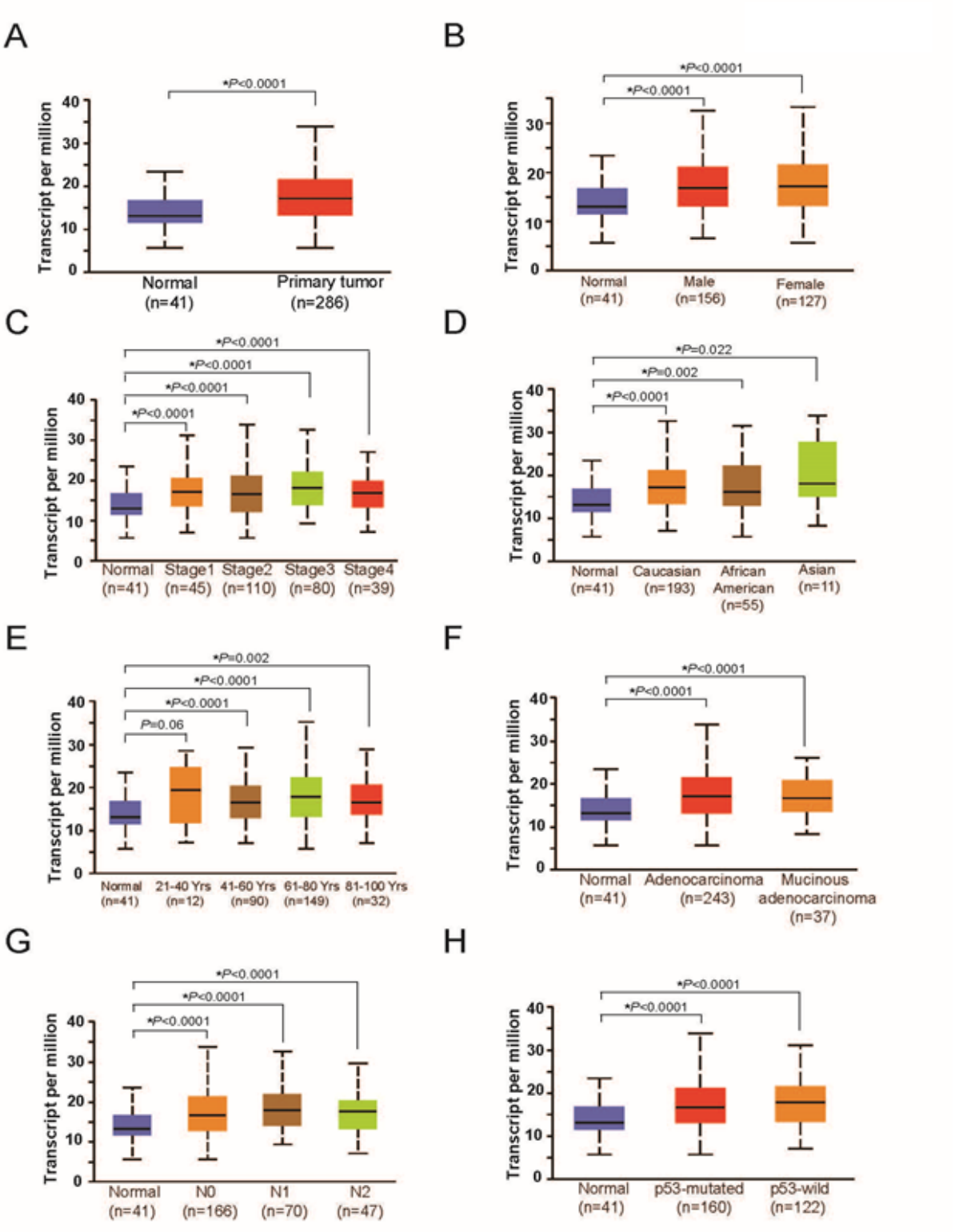

Figure 1

MICA RNA expression in CRC tissues. (A) Box plots showing relative expression of MICA mRNA in uninvolved tissues and CRC (Student's t-test). The mRNA expression levels were normalized as transcripts per million reads. (B) MICA expression in CRCs on the basis of patient sex, (C) various stages of CRC, (D) race, (E) age, (F) tumor histologic types, (G) nodal metastatic status, and (H) p53 mutation status (B-H, one-way ANOVA with Dunnett's multiple comparisons test). Pairwise comparisons relative to

normal tissue. CRC, colorectal cancer; MICA, MHC class I polypeptide-related sequence A; TCGA, The Cancer Gene Atlas.

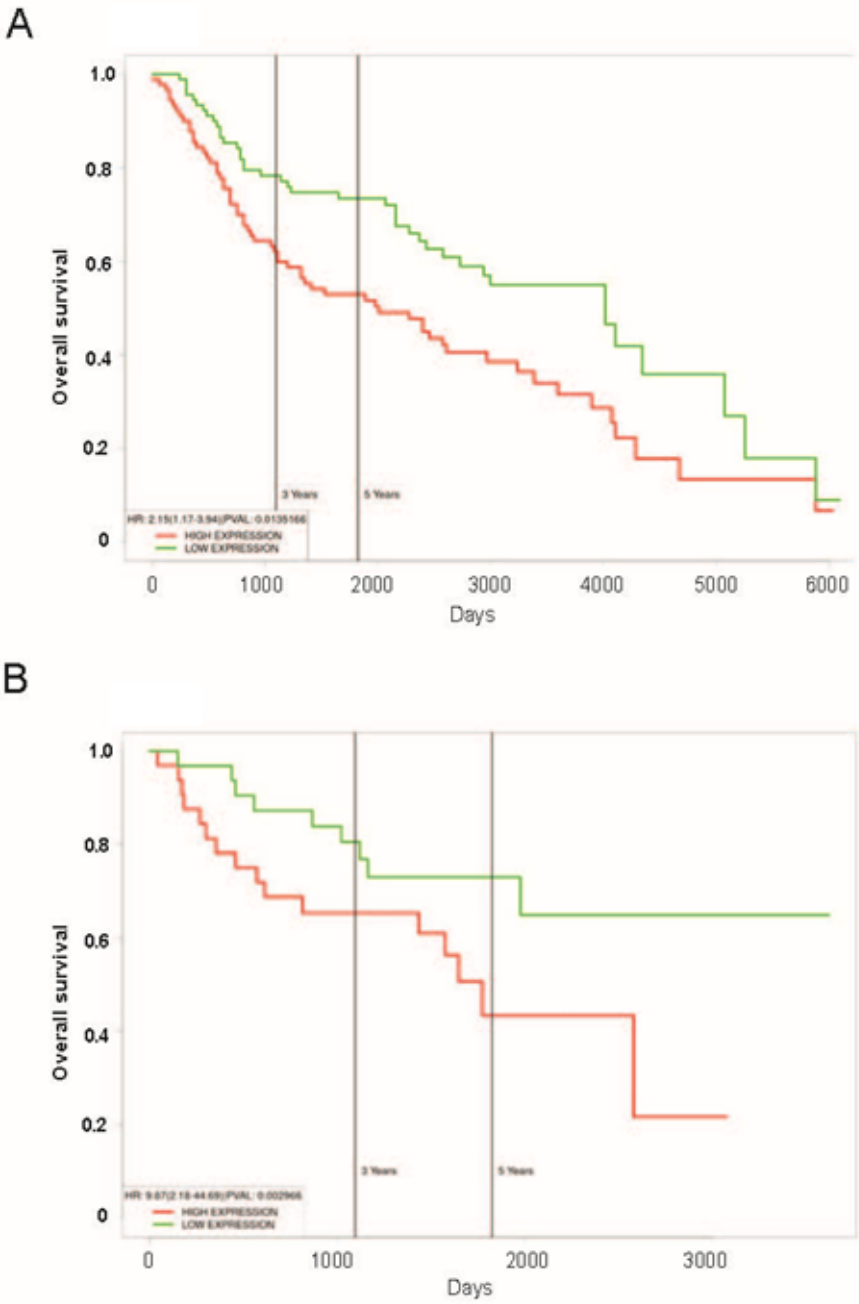

Figure 2

Survival analysis for patients with CRC according to the expression of MICA mRNA. Plots generated using the prognostic database PROGgeneV2 to analyze the datasets GSE41258 (A) and GS29621 (B), using the mean value as threshold. Poor prognosis was evident for patients with higher expression of MICA mRNA

in both datasets (log rank, P=0.014, HR: 2.15, 95% CI: 1.17-3.94 for GSE41258 and log rank, P=0.003, HR: 9.87, 95% CI: 2.18-44.69 for GS29621).

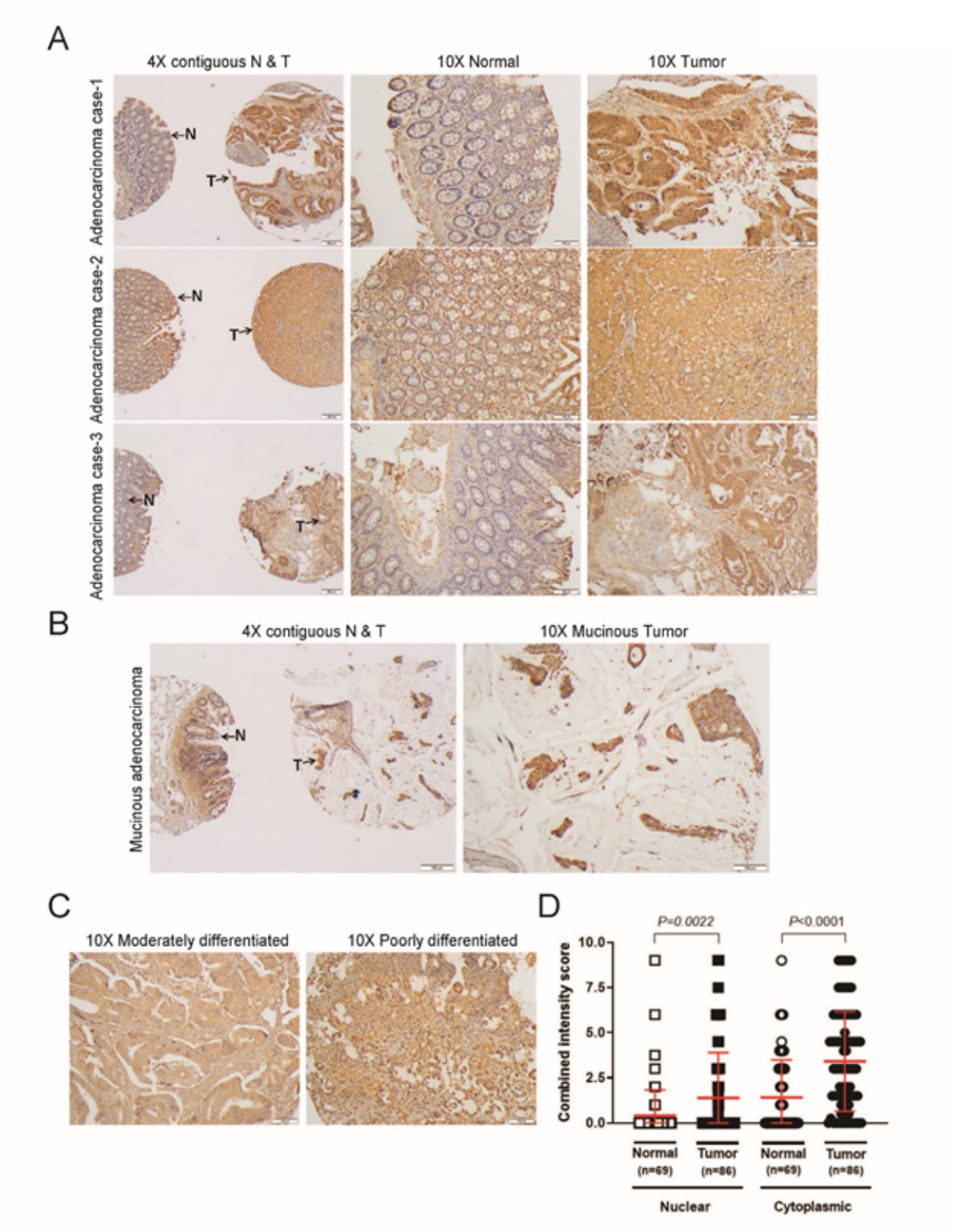

Figure 3

IHC staining of MICA. (A) Representative microphotographs from three different adenocarcinoma cases to show MICA staining in TMA tissues using IHC analysis. The 4X images (left panel) have contiguous normal (denoted by N) and tumor components (denoted by T) from adjacent sections of same patient

while 10X images are of normal (middle panel) and tumor (right panel) sections obtained from the TMAs. MICA glandular expression was nuclear and cytoplasmic in both normal and tumor tissue. Scale bar; 4X—100 μm, 10X—200 μm. (B) 4X image shows MICA staining in mucinous adenocarcinoma tissues with contiguous normal (denoted by N) section as well as tumor (denoted by T) component. (C) MICA staining in CRC tissues based on moderate and poor differentiation. (D). Stronger MICA immunoreaction was observed in tumor glandular cells relative to normal tissue, represented in the scatter plot. Mann-Whitney U test for non-matched data.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MICACRCSupplementarymaterials031521.docx