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Research Article

CHARACTERIZATION OF DIFFERENTIAL EXPRESSION PATTERNS OF THE EXTRACELLULAR PURINERGIC ENZYMES IN COLORECTAL CANCER

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Abstract: The aim of this study is to characterize tumor cell specific expression of purinergic ecto-enzymes CD39 and CD73, and to associate prognostic significance of these expression patterns in colorectal cancer (CRC) patients. Protein and gene expression of the target genes in various CRC cell lines were assessed via Western Blot (WB) analysis and Real Time PCR (RT-PCR). Additionally, tumor vs stromal cell expression of the target genes was analyzed from publicly available patient expression datasets. Finally, the correlation between CD39 and CD73 expression with patient prognosis was analyzed via The Cancer Genome Atlas (TCGA) datasets. In CRC cell lines, CD39 was found to be not expressed at all while CD73 was expressed extensively in most cell lines via WB and RT-PCR analyses. Patient microarray expression data confirmed the results from CRC cell lines that CD39 expression was very low in epithelial/tumor cells relative to other stromal cell types yet CD73 was expressed abundantly in every cell type within patient tumor samples. Interestingly, CD39 expression in patient tumors was correlated with favorable prognosis while CD73 expression was associated with worse prognosis. Although CD39 and CD73 are related enzymes involved in extracellular purinergic signaling, their expression patterns in tumor cells and prognostic effects in patients show opposing outcomes. Therefore, better insights in understanding the functional involvement of purinergic ecto-enzymes in colorectal tumor development is needed via further mechanistic studies.

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Özet: Bu çalışmanın amacı pürinerjik ekto-enzimler CD39 ve CD73'ün tümör hücresine özgü ifadesini karakterize etmek ve kolorektal kanser (KRK) hastalarında bu ifade örgülerinin prognostik öneminin anlaşılmasıdır. Çeşitli KRK hücre hatlarında hedef genlerin protein ve gen ifadesi, Western Blot (WB) ve Real Time PCR(RT-PCR) yoluyla değerlendirildi. Bunun yanında hedef genlerin tümör ve stromal hücrelerdeki ifadesi kamuya açık hasta ifade veri setleri vasıtasıyla analiz edildi. Son olarak, CD39 ve CD73 ifadesi ile hasta prognozu arasındaki ilişki The Cancer Genome Atlas (TCGA) veri setleri aracılığıyla analiz edildi. KRK hücre hatlarında CD39'un hiç ifade edilmediği, CD73'ün ise çoğu hücre hattında yoğun şekilde ifade edildiği WB ve RT-PCR analizleri yoluyla bulundu. Hasta mikrodizin ifade verileri, CD39 ifadesinin epitelyal/tümör hücrelerinde diğer stromal hücre tiplerine göre çok düşük olduğunu, ancak CD73'ün hasta tümör örneklerindeki her hücre tipinde bol miktarda ifade edildiğini KRK hücre hatlarından elde edilen sonuçları doğrular biçimde ortaya konuldu. İlginç bir şekilde, hasta tümörlerinde CD39 ifadesi iyi prognoz ile ilişkili olarak bulunurken CD73 ifadesi ise kötü prognoz ile ilişkili olarak gözlenmiştir. CD39 ve CD73 hücre dışı pürinerjik sinyal yolağında yer alan ilgili enzimler olmalarına rağmen tümör hücrelerindeki ifade örgüleri ve hasta prognozlarındaki etkilerinde birbirine zıt sonuçlara ulaşılmıştır. Sonuç olarak kolorektal tümör gelişiminde pürinerjik ekto-enzimlerin fonksiyonel katılımını daha iyi anlamak için daha ileri mekanistik çalışmalara ihtiyaç vardır.

Introduction

Colorectal cancers (CRCs) are among the most diagnosed and the deadliest malignancies in developing and developed countries. The number of newly reported cases throughout the world is continuously increasing and every year more than a million new patients are diagnosed with CRC (Sung *et al.* 2021). In recent years, CRC related deaths are no longer increasing as a result of the developments in diagnostic tools and cancer monitoring programs, but the 5-year-survival expectancy is still as low as 50% in CRC patients (Sung *et al.* 2021). The best

therapeutic option currently available is the surgical resection of the intestines which heavily depends on early diagnosis. Therapeutic options are very limited for the late stages and patient prognosis declines sharply after stage II (Rosa *et al.* 2015). Therefore, we urgently need more powerful tools for patient diagnosis and prognosis to choose the best treatment tailored for individual patients. This can be only achieved by better understanding of the signaling mechanisms leading to formation of various tumor subtypes seen in different patients.

Most CRCs are spontaneous but 10-15% of the cases can still be traced back to pre-existing inflammatory causes, such as Inflammatory Bowel Disease (IBD) (Rhodes & Campbell 2002). Even though the majority of CRC cases do not have pre-existing inflammation, they nevertheless show tissue infiltration by inflammatory cells (Grivennikov *et al.* 2010, Mantovani *et al.* 2008), leading to establishment of a chronic inflammatory tumor microenvironment (Terzić *et al.* 2010). The establishment of such an environment also depends on transcription factors such as Nuclear Factor kappa B (NF-κB) and Signal Transducer and Activator of Transcription 3 (STAT3), activities of which promote tumor cell survival and the expression of pro-inflammatory cytokines (Karin 2006, Ghosh & Hayden 2008, Bollrath *et al.* 2009,). In previous studies, we have provided further evidence for the involvement of tumor microenvironment for the initiation and progression of tumors (Göktuna *et al.* 2014, Göktuna *et al.* 2016). Especially, our and other's works have put forward how tumor cells modify inflammatory cells to mediate the tumor tolerance in various cancer models. Therefore, we need to know better about cell to cell interactions and how tumor cells modulate immune responses within the tumor microenvironment to generate alternative strategies for cancer therapy.

Extensive cell death, inflammation and hypoxia cause the leakage of vast amounts of ATP into extracellular matrix (ECM) in various physiologies including tissue damage, infections, autoinflammatory diseases and cancer (Cekic & Linden 2016). Since ATP has strong pro-inflammatory effects in tissues, extracellular purinergic metabolism enzymes CD39/ENTPD1 and CD73/NT5E must convert ATP quickly to adenosine which later contributes to the resolution of inflammation (Antonioli *et al.* 2013). Within tumor microenvironment two basic properties of adenosine have been defined; adenosine suppresses inflammation via activating adenosine receptors (such as Adora2a and Adora2b) on inflammatory cells and it also promotes tumor cell invasiveness and metastasis (Cekic & Linden 2014). Moreover, CD39, CD73 and their downstream adenosine receptors have been observed to be upregulated in tumor infiltrating immune cells (Antonioli *et al.* 2016). Since anti-inflammatory signaling and tumor tolerance are important for tumor cells to invade and metastasize (Parcesepe *et al.* 2016), these enzymes are highlighted in various cancers, including breast and colon, as prognostic markers (Stagg *et al.* 2010, Liu *et al.* 2012, Petruk *et al.* 2021). Particularly, both ecto-nucleotidases were shown to regulate invasive and metastatic abilities of breast tumor cells (Zhi *et al.* 2007, Zhou *et al.* 2007). However, little is known about the involvement of these proteins in colorectal tumor development (Wu *et al.* 2016, Xie *et al.* 2017, Hajizadeh *et al.* 2020). This is especially interesting that CRC is unique among others due to involvement of microflora within the intestinal tumor microenvironment. Even less is known if these expressions are due to tumor cells, endothelial cells or immune cells (Roufas *et al.* 2021). Therefore, in this study, we aim to evaluate cell specific expression patterns of extracellular purinergic signaling components, CD39 and CD73, to better identify how these molecules are differentially expressed within the tumor microenvironment. Finally, this knowledge will eventually help us to develop better diagnostic, prognostic and therapeutic tools for tackling CRC.

Materials and Methods

Cell lines and tissue culturing

SW480, HT-29, RKO, Caco-2 and SW48 cells (ATCC, USA) were cultured in low glucose DMEM (Biowest, France); SW48 cells were also cultured in L15 (Sigma, USA) where indicated; DLD-1 and HCT116 cells (ATCC, USA) were maintained in McCoy 5A medium (Biowest, France); SW620 cells (ATCC, USA) were cultured in RPMI1640 (Biowest, France) media. All these media were supplemented with 10% FCS (Biowest, France), 1% L-glutamine (Lonza, Switzerland) and 1% penicillin-streptomycin (Lonza, Switzerland).

Gene expression analysis via Western blotting, RT-PCR

Western Blotting

All experimental procedures were carried out as previously described (Serkan Ismail Göktuna *et al.* 2016). Briefly, crude cell lysates from above mentioned CRC cell lines were collected. After boiling the lysates in a loading buffer for 5 mins, equal amounts of total protein extracts were loaded to 8-12% SDS-PAGE gels (depending on expected band sizes) for separating protein according to their molecular weight. Then these gels were blotted onto PVDF membranes (Millipore, USA) where target proteins were coupled with specific primary and HRP-linked secondary antibodies. Finally, specific proteins were visualized as positive bands by the use of chemiluminescence reagent (Pierce/Thermo, Germany) and recorded in Amersham Imager 600 (GE Healthcare, USA). Antibodies used are anti-CD39 (BU61, SC-65262, SCBT, USA); anti-CD73 (D7F9A, #13160, Cell Signaling, USA); anti-beta-actin (SC-8432, SCBT, USA). All antibodies were diluted as 1:1000. All experiments were repeated at least three times and representative results were presented.

Gene Expression Analysis via RT-PCR

Total RNAs were extracted using the E.Z.N.A Total RNA kit (Omega Biotek, USA) and cDNAs were synthesized by the use of iScript cDNA Synthesis kit (Biorad, USA) as instructed by the producers. RT-PCR analysis was performed by the use of LightCycler

FastStart DNA Master SYBR Green (Roche, Germany) on a Lightcycler 480 Real-Time PCR System (Roche, Germany) as described previously (Göktuna 2022). The primer sequences hCD39F 5′- AGC AGC TGA AAT ATG CTG GC -3′; hCD39R 5′-GAG ACA GTA TCT GCC GAA GTC C-3'; hCD73F 5′-ATT GCA AAG TGG TTC AAA GTC A-3′; and hCD73R 5′-ACA CTT GGC CAG TAA AAT AGG G-3′ were used. All experiments were repeated at least three times and representative results were presented.

Bioinformatic Analyses

Microarray data related to FACS separated CRTs were acquired from GSE39396 dataset downloaded from GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) and Robust Multichip Average (RMA) normalized according to "User Guide" instructions as described previously (Demirkol *et al.* 2017). Then the data was plotted and analyzed in GraphPad Prism 6 Software. For gene expression in tumor (275 samples) vs normal tissues (349 samples), TCGA RNA-Seq data publicly available at GEPIA website (http://gepia.cancer-pku.cn/) were utilized. In this analysis, used parameters were log_2FC cut off:1; *P*-value 0.05 (95% CI); tumor-dataset: COAD (TCGA); normal-dataset: TCGA normal and GTEx data; plotted in log_2(TPM+1) for log-scale. For patient survival analysis, CRC patient microarray data (Smith *et al.* 2010, Sveen *et al.* 2011) available in PRECOG database were used (Gentles *et al.* 2015). In PRECOG website meta-Z analyses were performed as previously described (Storey & Tibshirani 2003). Survival curves were plotted and derived from the PRECOG website (Stanford University). Briefly, microarray data were used to plot overall survival (OS) Kaplan-Meyer plots (KM-plots) as median expression cut-off (50% high vs. 50% low); hazard ratio (HR) were calculated according to Cox PH model (HR>1 associated with worse prognosis and HR<1 was associated with good prognosis); *P*-value of 0.05 (log-rank test) and lower were considered significant; for median survival months off survival were used in the graphs. We fully acknowledge the use of online tools available at GEPIA and PRECOG websites for associating patient expression data with the pathways of interest. All these data are publicly available and obtained as described above.

Statistics

For statistical analysis of the RT-PCR expression data, Student's t-test was used. In all expression graphs, error bars were plotted according to one standard deviation; *P* values less than 0.05 were considered significant (95% confidence interval (CI)). * $P<0.05$ (95% CI); ** $P<0.01$ (99% CI); *** $P<0.001$ (99.9% CI). For analysis of patient microarray expression data, Wilcoxon matched-pairs signed test was used, p values less than 0.05 was considered significant (95% confidence interval (CI)). All statistical analyses were performed by using the Prism 6 Software (Graphpad, USA). In Kaplan-Meyer survival graphs, log-rank test was used, *P* values less than 0.05 were considered significant (95% CI). All test results were available from the PRECOG database.

Results

In this study, gene expression profiles for purinergic ecto-enzymes CD39 and CD73 were analyzed in CRC cell lines and patient sample data available in public databases. First, CRC cell lines available in our lab (HT-29, DLD-1, SW480, RKO, HCT116, SW620, SW48 and Caco-2) were grown in their respective media and then cells were harvested to obtain protein, or mRNA extracts (Figs 1-2). Protein and mRNA extracts from unstimulated Jurkat cells were also obtained and included in gene expression or protein expression analyses (Fig. 1). First, CD39 and CD73 protein levels in CRC and hematopoietic cells (Jurkat) were compared via Western Blot (WB) analysis by the use of specific antibodies (Fig. 1a). WB analyses have shown that CD73 was widely expressed in certain CRC cell lines (namely SW48, HT-29, DLD-1, SW480 and HCT116) while CD39 protein expression was mostly absent in CRC cell lines, with the exception of SW48 (Fig. 1a). These expression profiles were also confirmed in RT-PCR analyses (Figs 1b-c) where CD73 mRNA expression was again abundant (1.5-4× of the control cells) in the same CRC cell lines ($\Delta\Delta C_T$ of $8.0*10^{-3}$ to $2.3*10^{-2}$) relative to hematopoietic cells ($\Delta\Delta C_T$ of $5.3*10^{-3}$) as in the case of WB results. However, CD39 expression was largely absent (0.025-0.08× of the control cells) in CRC cells ($\Delta\Delta C_T$ of $2.0*10^{-4}$ to $6.3*10^{-4}$), except Caco-2 cells ($\Delta\Delta C_T$ of $8*10^{-3}$), relative to hematopoietic control Jurkat cells ($\Delta\Delta C_T$ of $1.6*10^{-2}$) (Figs 1b-c). Although unstimulated hematopoietic cells were not the best control to compare expression levels, CD73 mRNA and protein were robustly and abundantly expressed in above mentioned CRC cell lines while CD39 expressions were widely absent with the above mentioned exceptions. However, no confirmation of these WB vs. RT-PCR results was possible in CD39 expressing cells since CD39 mRNA was absent in SW48 cells and CD39 protein was absent in Caco2 cells. Similar results were obtained in FACS analyses carried out with several CRC cell lines. CD39 surface expression was absent while CD73 was abundantly expressed in SW480, HT-29, DLD-1 and HCT116 cells (unpublished data). Therefore, CD73 was found to be expressed in most of the CRC cells lines yet CD39 expression was lacking in almost all CRC cell lines we analyzed via different means.

Finally, CD39 and CD73 expression patterns were analyzed in publicly available datasets (Fig. 2). First, microarray data generated from FACS separated colorectal tumors (GSE39396) were analyzed and it was observed that CD39 expression was much lower in $EpCAM^+$ epithelial/cancer cells (0.49-0.65× of other cells, log_2 of expression values were 3.58-4.17 in $EpCAM^+$ cells whereas log_2 of expression values were 6.01-8 in all other stromal cells, for all cases $P<0.05$ (95% CI) and significant, according to Wilcoxon matched-pairs signed rank test) in tumor than any other cell type (endothelial cells, fibroblasts and hematopoietic cells) in the tumor stroma (Fig. 2a).

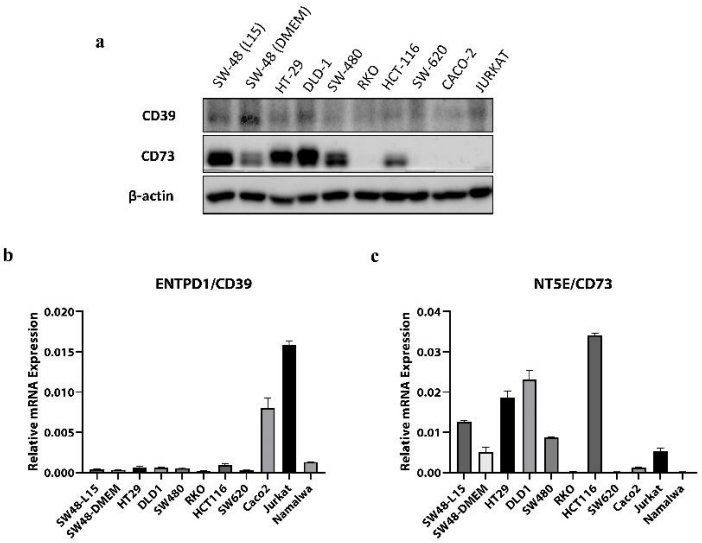

Fig. 1. a. CD39 and CD73 protein expressions from indicated CRC cell lines were measured via WB. β-actin WB was used as loading control, **b.** CD39 and **c.** CD73 mRNA expressions from indicated CRC cell lines were measured via RT-PCR. GAPDH expressions were used as housekeeping control for both cases. Statistical significance was evaluated via Student's t-test, error bars were plotted according to one standard deviation (95% CI).

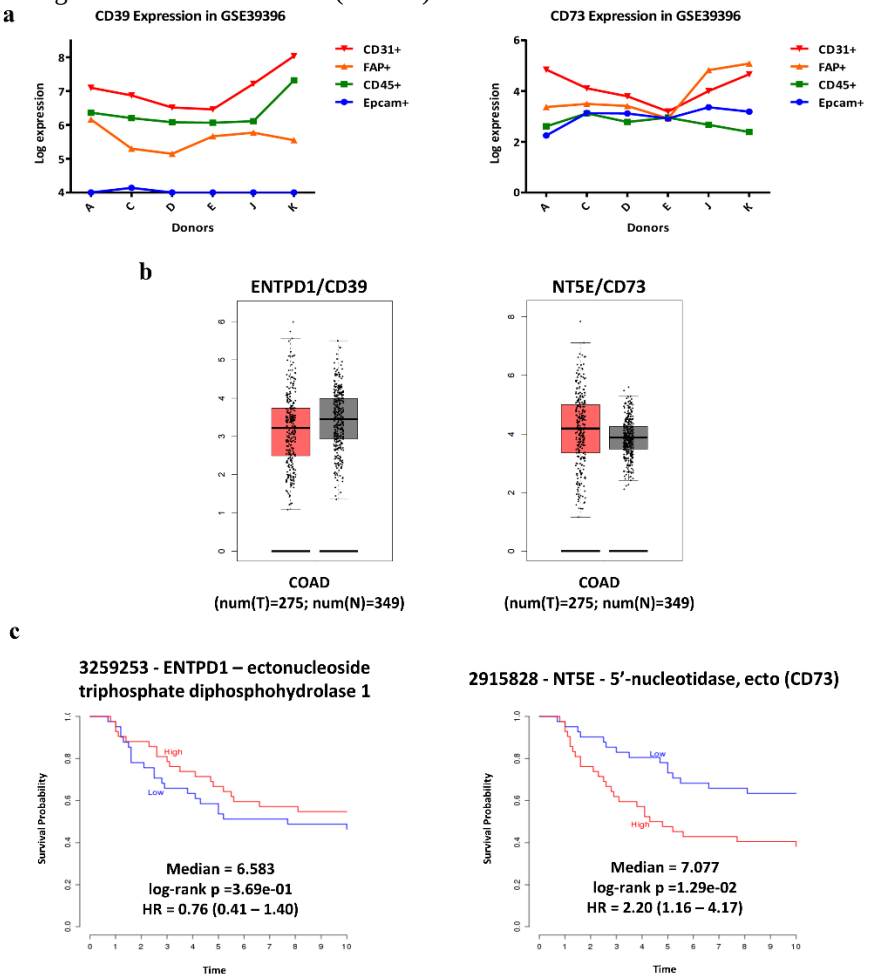

Fig. 2. a. Cell surface expression of ENTPD1/CD39 (left) and NT5E/CD73 (right) in FACS-sorted cells ($CD31^+$ for endothelial; FAP^+ for fibroblast/stromal cells; $CD45^+$ for immunocytes; $Epcam^+$ for epithelial cells) obtained from 6 CRC tumors (donors A-K) in GSE39396 dataset. One representative probe set for each gene was shown, **b.** tumor vs. normal tissue ENTPD1/CD39 (left) and NT5E/CD73 (right) mRNA expression levels in TCGA-COAD (colon adenocarcinoma) patient sample database (differential expression graphs generated in GEPIA), **c.** Overall survival (OS) in CRC patient cohorts available in the PRECOG database according to CD39 (left) and CD73 (right) expression in the tumor samples. Only high CD73 expression is significantly associated with poor prognosis in CRC samples. Statistical significance was evaluated via log-rank test, p values less than 0.05 was considered significant (95% CI).

However, similar to *in vitro* findings, CD73 expression in the epithelial/tumor cells were as abundant as other cells in the tumors (0.59-1.26× of other cells, $\log_2$ of expression values were 2.24-3.35 in $EpCAM^+$ cells whereas $\log_2$ of expression values were 2.38-5.08 in all other stromal cells, for all cases $P>0.05$ and differences were not significant (95% CI), according to Wilcoxon matched-pairs signed rank test). Therefore, CD73 is highly expressed in cancer cells and may be relevant with their tumorigenic potentials. Moreover, CD39 vs CD73 expression in normal vs tumor samples of CRC patients were analyzed in TCGA datasets (Fig. 2b) which was showing reduced CD39 in tumor while increasing CD73 in tumors than in normal tissues (differences were significant, $P<0.05$ in both cases). Finally, the prognostic potential of the purinergic ecto-enzymes was analyzed by the use of PRECOG patient microarray expression datasets (Fig. 2C). In PRECOG colorectal cancer patient survival datasets, meta-Z analysis showed that CD39 is found to be slightly but not significantly associated with favorable prognosis (Z-score of -0.34, $P>0.05$, not significant) while CD73 expression was found to be highly associated with worse prognosis (Z-score of 3.18 or $P<0.001$) (results are not shown). Among 6 different datasets within PRECOG, one representative survival graph was presented (Fig. 2c) in which CD39 was slightly but not significantly associated with good prognosis (HR=0.76; n.s., $P=0.369$) while CD73 was significantly associated with worse prognosis (HR=2.20; *, $P=0.0129$) (Smith *et al.* 2010, Sveen *et al.* 2011). Collectively, these results indicate that CD39 and CD73 may be involved differentially in CRC development.

Discussion

Although CD39 and CD73 have been defined as prognostic markers in various cancers (X.-R. Wu *et al.* 2012), there are only a few studies describing their mechanism in breast tumor invasiveness (Wang *et al.* 2008, Choi *et al.* 2020). In CRC, there are no mechanistic studies to prove their oncogenic or metastatic potential. Hence, functional tumor models need to be developed to understand the mechanism of extracellular adenosine metabolism involvement in colorectal cancer dissemination.

Recently, a number of reports claimed that purinergic receptors help to reduce tumor immunogenicity and prevent anti-tumor response by the inflammatory cells (Antonioli *et al.* 2013, Antonioli *et al.* 2016, Cekic & Linden 2016). These receptors are not only expressed in inflammatory cells but also highly expressed in endothelial cells (Feng *et al.* 2011). As a result, their overexpression and anti-inflammatory signaling are enriched, which leads to the blocking of anti-tumor immune response to promote tumor progression and metastasis. This phenomenon has been shown to be true especially in studies with breast cancer yet it needs to be proven in other cancer types (Stagg *et al.* 2010, Stagg *et al.* 2011, Stagg *et al.* 2012). Especially in breast tumor development, CD73 and purinergic signaling may be involved in the activation of several molecular pathways (Soleimani *et al.* 2019, Vasiukov *et al.* 2020). Consequently, CD73 has been suggested to be a target for immunotherapy in mouse models of breast cancer (Liu *et al.* 2021). There are reports showing that CD39 enzymatic activity may be targeted for alleviating tumor cell-mediated immunosuppression (Bastid *et al.* 2015).

Considering elevated purinergic signaling levels in colon cancer samples, we strongly suppose that purinergic ecto-enzymes can be responsible for a similar anti-inflammatory/immunosuppressive role in colorectal tumorigenesis (X.-H. Liu *et al.* 2020). Given their immunosuppressive powers, it would not be surprising to see that these proteins are highly expressed in the colorectal tumor microenvironment (Liu *et al.* 2012). Interestingly, according to our analyses, only CD73 is highly expressed in tumor cells while CD39 expression is totally absent in almost all colorectal cancer cells. This observation was also supported by a previous report (Bastid *et al.* 2015). This study also shows that CD39 expression is absent in HCT116 cells and lower in colorectal cancer patient tumor samples relative to matched normal tissues, further supporting our in vitro and in silico findings. Lower CD39 expression in colorectal tumors is quite different than other cancers analyzed in the same study where breast, lung, melanoma, ovary, pancreas, prostate, kidney, lymphoid, liver, or head & neck, testis and thyroid cancers show higher CD39 expression in tumors. On the other hand, we observed that only CD73 expression is correlated with worse prognosis yet CD39 expression is lower in tumors and high CD39 expression is correlated with favorable patient prognosis in CRC. These opposing expression patterns for two related purinergic ecto-enzymes is a very surprising finding given that these enzymes are both involved in the same biological pathway. While CD39 expression is limited to stromal cells, CD73 can also be expressed highly in tumor cells. The role of CD73 expression may be context dependent in colorectal cancer. While some studies associate stromal CD73 expression with higher tumorigenic potential in colorectal cancer (Wu *et al.* 2016, Liu *et al.* 2020, Yu *et al.* 2020), other studies suggested that CD73 expression may have contrasting results in colorectal tumor metastasis (Tokunaga *et al.* 2019, Matsuyama *et al.* 2010, Messaoudi *et al.* 2020).

In CRC studies with the cell lines, tumor cell specific CD73 expression was found to be essential for tumor growth *in vitro* and *in vivo* (Wu *et al.* 2016). However, in a colitis associated cancer model, use of CD73 specific inhibitor reduced the tumor burden (Liu *et al.* 2020). On the contrary, another report suggested that tumor cell specific CD73 expression is dispensable for colorectal tumor growth while stroma specific (mesenchymal cells) CD73 expression was essential for CD73-driven tumor growth (Yu *et al.* 2020). The involvement of CD73 has even more interesting and contrasting outcomes depending on the model. These studies collectively support a stroma-associated anti-inflammatory role for CD73 in tumor microenvironment. Similarly, another

study put forwards that CRC-derived extracellular vesicles may drive the immunosuppressive activity of CD73 expressed in cancer cells which may be targeted via a bispecific antibody (CD73×EpCAM) (Ploeg *et al.* 2021). This study further strengthens the immunosuppressive role for CD73 in colorectal tumor microenvironment. While previous reports suggested that CD73 absent tumor cell lines are more aggressively metastatic (Matsuyama *et al.* 2010), the results from patients with liver metastasis show that CD73-high patients have worse prognosis (Messaoudi *et al.* 2020). Though the latter study did not include whether CD73 is expressed by tumor cells or by the stroma, we can conclude that CD73 expression overall makes them more aggressive and metastatic (Messaoudi *et al.* 2020). However, these conclusions do not exclude the possibility of CD73 having cell type specific effects in CRC development. This was also shown to be important in breast tumorigenesis (Gong *et al.* 2020). Indeed, our preliminary data shows that CD73 expression in immune or stroma-low tumors may have totally different prognostic outcomes (unpublished data). We hope to elaborate about this phenomenon in a later report.

The dual characteristic of expression patterns and prognostic effects by these two seemingly similar enzymes make us think that their biology may be much more complex in colorectal cancer than previously thought. Recent literature and our findings suggest that these purinergic ecto-enzymes may be exclusively expressed in different cells within the tumor microenvironment. Moreover, their involvement in colorectal tumorigenesis may be different depending on the cancer stage or the context within tumors. Consequently, we may need more elaborative functional analyses and cell specific models to better dissect involvement of purinergic ecto-enzymes in tumor development, metastasis and patient prognosis.

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Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Conflict of Interest: The author has no conflicts of interest to declare.

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Data Availability: All the genomic data presented here are freely available at online databases or repositories. Gene expression data was obtained from GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi); clinical data was obtained from Array Express (http://www.ebi.ac.uk/arrayexpress). Patient prognosis and gene expression data was obtained from GEPIA (http://gepia.cancer-pku.cn/) or PRECOG (https://precog.stanford.edu/).

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