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Molecular Regulators of Small Extracellular Vesicle Biogenesis in Colorectal Cancer: Associations with Tumor Expression, Plasma Levels, and Patient Survival

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ABSTRACT

An expanding body of research has emphasized the involvement of small extracellular vesicles (sEVs), particularly exosomes, in colorectal cancer (CRC). Despite this, information regarding the prognostic or clinical relevance of molecules involved in sEV production in CRC remains scarce. In this project, we examined the expression patterns of key genes governing sEV formation and explored how these relate to plasma sEV quantities—measured with a double-sandwich ELISA—as well as clinical outcomes in CRC patients. Our findings indicate that mRNA levels of RAB27A, RAB27B, RAB2B, and RAB3B were significantly reduced in tumor samples when compared with adjacent non-cancerous tissues ($p < 0.001$, $p = 0.009$, $p = 0.011$, and $p < 0.001$, respectively). Moreover, elevated tumor expression of RAB27A, RAB27B, RAB9A, RAB11B, and STX1A corresponded with improved 5-year survival ($p = 0.038$, $p = 0.015$, $p = 0.008$, $p = 0.002$, and $p = 0.028$, respectively). Patients with adenomas also exhibited lower total plasma sEV levels than healthy controls ($p = 0.026$). However, CRC patients showed no significant differences in either total or tumor-originating sEV concentrations ($p = 0.885$ and $p = 0.330$, respectively). Overall, our results suggest that sEV biogenesis may play an important role in CRC progression, with RAB27A, RAB27B, RAB9A, RAB11B, and STX1A emerging as potential markers of survival.

Keywords: Colorectal cancer, SEVs, Exosomes, Survival, Expression

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Introduction

Over the past ten years, interest in small extracellular vesicles (EVs)—including exosomes—within oncology and particularly CRC has risen sharply. These sEVs are nanosized (30-150 nm) membrane-bound vesicles carrying DNA, mRNAs, miRNAs, lncRNAs, and protein cargo [1-4]. While once considered simple metabolic by-products, they are now recognized as critical mediators of intercellular signaling, antigen handling, and the transport of biological molecules [5].

A wide range of investigations have shown that malignant cells release larger quantities of sEVs both in vitro and in vivo compared with healthy cells. These cancer-derived vesicles promote pathways that enhance cell growth and angiogenesis, contribute to tumor infiltration, and support immune evasion [6, 7]. Additional reports link sEVs to tumor progression, remodeling of the tumor microenvironment, metastatic niche preparation, distant spread, and therapy resistance [5].

sEVs originate inside the cell through a multistep maturation process. First, early endosomes form; these then develop into late endosomes, which subsequently generate multi-vesicular bodies (MVBs) containing intraluminal vesicles (ILVs). MVBs may then either undergo lysosomal degradation or fuse with the plasma membrane to release ILVs as sEVs [8, 9]. This pathway depends on numerous proteins and complexes and is not yet fully mapped. The ESCRT machinery is central to ILV formation and cargo selection [10], although ESCRT-independent routes also exist [11]. Several additional molecules—including Rab GTPases and syntaxins, which

function within SNARE complexes—are involved in MVB formation, selective loading of cargo, and ultimate vesicle release [12].

In CRC specifically, sEVs have been linked to accelerated tumor growth, metastatic behavior, EMT processes, angiogenic signaling, and modulation of the tumor microenvironment [13]. They have also been tied to therapeutic resistance [14] and are being explored as delivery vehicles for CRC therapies [15, 16]. Despite this, there is limited evidence evaluating the clinical impact of genes that regulate sEV production. The present study analyzes the expression of major sEV-associated genes and investigates their relationship with sEV plasma levels and patient prognosis.

Materials and Methods

Gene selection

To identify genes highly relevant to exosome formation in CRC, we extensively reviewed publicly available studies (PubMed and Google Scholar) using gene names associated with sEV production and CRC-related terminology, ultimately narrowing the field to 9 studies. We also incorporated data from TCGA and GTEx using the GEPIA platform for comparing gene expression in cancerous versus non-cancerous tissues [17]. Based on differential expression patterns between CRC and normal samples, 8 genes were chosen as the most promising candidates.

To assess the influence of these genes on patient survival, we utilized GEPIA [17] and the KMplotter database, focusing specifically on CRC cohorts [18]. The final gene panel included: RAB2B (Ras-Related Protein Rab-2B), RAB3B (Ras-Related Protein Rab-3B), RAB9A (Ras-Related Protein Rab-9A), RAB11B (Ras-Related Protein Rab-11B), RAB27A (Ras-Related Protein Rab-27A), RAB27B (Ras-Related Protein Rab-28B), STX1A (Syntaxin 1A), and VAMP7 (Vesicle-Associated Membrane Protein 7).

Patients and samples

This investigation was conducted prospectively and received authorization from the Scientific Committee and the Ethics and Research Committee of the University Hospital of Patras, Greece (451/30/9/2016). Written consent was obtained from each individual taking part. The study population consisted of 121 CRC patients, 39 individuals with adenomas, and 39 healthy controls. Their demographic information and, for the CRC group, clinical characteristics, appear in **Table 1**. All participants were evaluated and treated within the same institution, specifically across the Departments of Gastroenterology, Surgery, and Oncology.

Tumor tissue together with matched, non-cancerous surrounding tissue was collected from 109 CRC patients and immediately placed in RNAlater RNA Stabilization Reagent (Sigma-Aldrich, St. Louis, MO, USA) and frozen at −80 °C. Diagnoses were confirmed by histological examination.

Blood was drawn from 65 CRC patients, all 39 adenoma patients, and all 39 healthy volunteers either before colonoscopy, ahead of tumor removal surgery, or after surgical treatment. Samples were collected in K2EDTA Vacuette tubes (Greiner BioOne, Frickenhausen, Germany). Plasma was separated no later than 2 h after collection by centrifuging at 1500× g for 20 min at 4 °C, then stored at −80 °C.

Table 1. Demographic and clinical profile of CRC/adenoma cases and healthy individuals. N/A = not available.

Demographic/Clinicopathological Characteristics	Cancer Patients	Adenoma Patients	Healthy Individuals
Gender			
Male	72 (59.5%)	26 (66.6%)	13 (33.3%)
Female	49 (40.5%)	13 (33.3%)	26 (66.6%)
Age Group			
≤66	40 (33.1%)	26 (66.6%)	24 (61.5%)
>66	81 (66.9%)	13 (33.3%)	15 (39.5%)
Stage			
In situ	7 (5.8%)	-	-
I	1 (0.8%)	-	-
II	58 (47.9%)	-	-
III	41 (33.9%)	-	-
IV	8 (6.6%)	-	-

N/A	6 (5%)	-	-
Grade			
I	15 (12.4%)	-	-
II	80 (66.1%)	-	-
III	5 (4.1%)	-	-
N/A	21 (17.4%)	-	-
Primary Site			
Right Colon	42 (34.7%)	-	-
Left Colon and Sigmoid	34 (28.1%)	-	-
Rectum	39 (32.2%)	-	-
N/A	6 (5%)	-	-
Lymph Node metastasis			
No	68 (56.2%)	-	-
Yes	45 (37.2%)	-	-
N/A	8 (6.6%)	-	-
Distant metastasis			
No	98 (81%)	-	-
Yes	9 (7.4%)	-	-
N/A	14 (11.6%)	-	-

RNA isolation and cDNA synthesis

RNA extraction from 109 CRC tissues and 60 adjacent non-malignant paired samples was performed with the PerfectPure RNA Tissue Kit (5Prime, Hamburg, Germany). DNase treatment (Ambion, Austin, TX, USA) followed. RNA quantity was assessed using a Nanodrop-1000 (NanoDrop, Fisher Thermo, Wilmington, DE, USA), and samples were subsequently kept at −80 °C.

For cDNA synthesis, 4 μg of each RNA sample was reverse transcribed using 100 U Superscript III Reverse Transcriptase, 300 ng random primers (Foundation for Research and Technology-Hellas, Crete, Greece), and 5 nM dNTPs (Enzyquest, Crete, Greece) in a 50 μL reaction. A no-enzyme control ensured the absence of DNA. Reactions in a C1000 Touch thermal cycler (Bio-Rad) followed this protocol: 25 °C for 5 min, 50 °C for 60 min, 70 °C for 15 min. Final cDNA products were diluted to 25 ng/μL and frozen at −20 °C.

Gene expression quantification

Expression of RAB2B, RAB3B, RAB9A, RAB11B, RAB27A, RAB27B, STX1A, and VAMP7 was quantified via qRT-PCR using custom primers and probes created by the research team. Oligonucleotides were synthesized by Metabion International AG (Martinsried, Germany) and are listed in **Table 2**.

Each 20 μL PCR reaction contained:

- 3 μL cDNA
- 300 nM forward primer
- 300 nM reverse primer
- 100 nM probe
- 10 nM reference dye (ThermoFischer Scientific)
- 200 μM dNTPs (ThermoFischer Scientific)
- 1 U Platinum Taq DNA Polymerase
- 1× buffer (containing 16.6 mM ammonium sulfate, 67 mM Tris pH 8.8, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol)

PCR was run on a StepOne Plus (ThermoFischer Scientific) under the following program: 95 °C for 10 min, then 45 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s.

Relative expression was calculated using LinRegPCR [19]. Normalization was performed using IPO8 (Importin 8) as the reference gene [20, 21]. All analyses were carried out blinded to sample type and participant identity.

Table 2. Primer and probe sequences used for qRT-PCR.

Gene	Forward 5′-3′	Reverse 5′-3′	Probe 5′	Size (bp)
RAB2B	CAAATCTGGGATACGGCTG	GTACACCAGCAG TGCTC	FAM/TCCTTC CGTTCTATCACCCGT/BHQ	87
RAB3B	ACGAGAAGCGGGTGAAAC	TAATAGGCTGTTGTGATGGTC	FAM/CTGGGACACAGCTGGGCA/BHQ	76
RAB9A	TCTCTCTGTCCTCATTGC	CTCAAAAGCTTCAAGAACCC	FAM/TCGCGGCCACACGAAAGA/BHQ	89
RAB11B	TTCAAAGTGGTGCTCATCG	TCCAGGTTGAACTCGTTG	FAM/AGGCGTGGGCAAGAGCAA/BHQ	83
RAB27A	GCACTCGCAGAGAAATATGG	TGCTTGGCTTATGTTTGTCC	FAM/CCCTACTTTGAAACTAGTGCTGCCA/BHQ	72
RAB27B	ACCAGTCAACAGAGCTTC	ATATCTGGATTTTCACAATAAGC	FAM/GAAACTGGATGAGCCAACTGCA/BHQ	80
VAMP7	AACTACCAGCAGAAATCTTG	ATGAACACAATTGATACGATG	FAM/AGCCATGTGTATGAAGAACCTCAA/BHQ	87
STX1A	CATTGACAAGATCGCAGAG	CTCCTCCTTCGTCTTCTC	FAM/GAGGAGGTGAAGCGGAAGCA/BHQ	94

Plasma sEV quantification

Total plasma sEV levels for 65 CRC patients, 39 adenoma patients, and 39 healthy participants were measured with the ExoTest double-sandwich ELISA kit (HansaBioMed Life Sciences, Tallinn, Estonia), which employs CD9 as the detection marker [22]. Tumor-associated sEV levels for the same CRC cohort were assessed using the corresponding tumor-specific ExoTest kit. This assay enriches vesicles expressing TM9SF4, a tumor-associated surface protein patented by HansaBioMed, with detection again performed through CD9.

For each participant, 500 µL plasma was subjected to triple centrifugation. Duplicate wells on a 96-well plate were loaded with 100 µL of the sample and incubated for 15 h at 4 °C. Afterward, a chromogenic substrate was applied for 5 min. A standard curve enabled the determination of absolute exosome concentration. The kits' validity has previously been confirmed through TEM imaging [22].

Statistical analysis

All statistical work was performed using IBM SPSS Statistics 21.0 (Armonk, NY, USA). Kruskal-Wallis tests compared gene expression across malignant and non-malignant tissues. Paired comparisons between matched tissues or within-patient gene and sEV levels used the Wilcoxon signed-rank test. Associations with clinicopathological features were evaluated using Mann-Whitney and Kruskal-Wallis tests. Pearson correlation assessed relationships among gene expression values and sEV concentrations.

Survival analyses used Kaplan-Meier curves with log-rank testing. The prognostic contribution of gene expression and sEV measurements was analyzed using Cox regression. Overall survival (OS) for CRC patients was determined after 60 months using medical records or direct follow-up by phone or in person. A p-value < 0.05 was considered statistically significant.

Results and Discussion

Reduced gene expression in malignant tissue compared with adjacent normal tissue

Expression profiling of RAB2B, RAB3B, RAB9A, RAB11B, RAB27A, RAB27B, STX1A, and VAMP7 was performed across 109 colorectal tumors and 60 paired non-malignant, tumor-adjacent samples. Markedly lower mRNA abundance was detected in tumors for RAB27A, RAB27B, RAB2B, and RAB3B ($p < 0.001$; $p = 0.009$; $p = 0.011$; $p < 0.001$, respectively; **Figure 1**). In contrast, RAB9A, RAB11B, STX1A, and VAMP7 did not exhibit

meaningful differences between malignant and neighboring non-tumorous tissues (p = 0.300; p = 0.243; p = 0.646; p = 0.472).

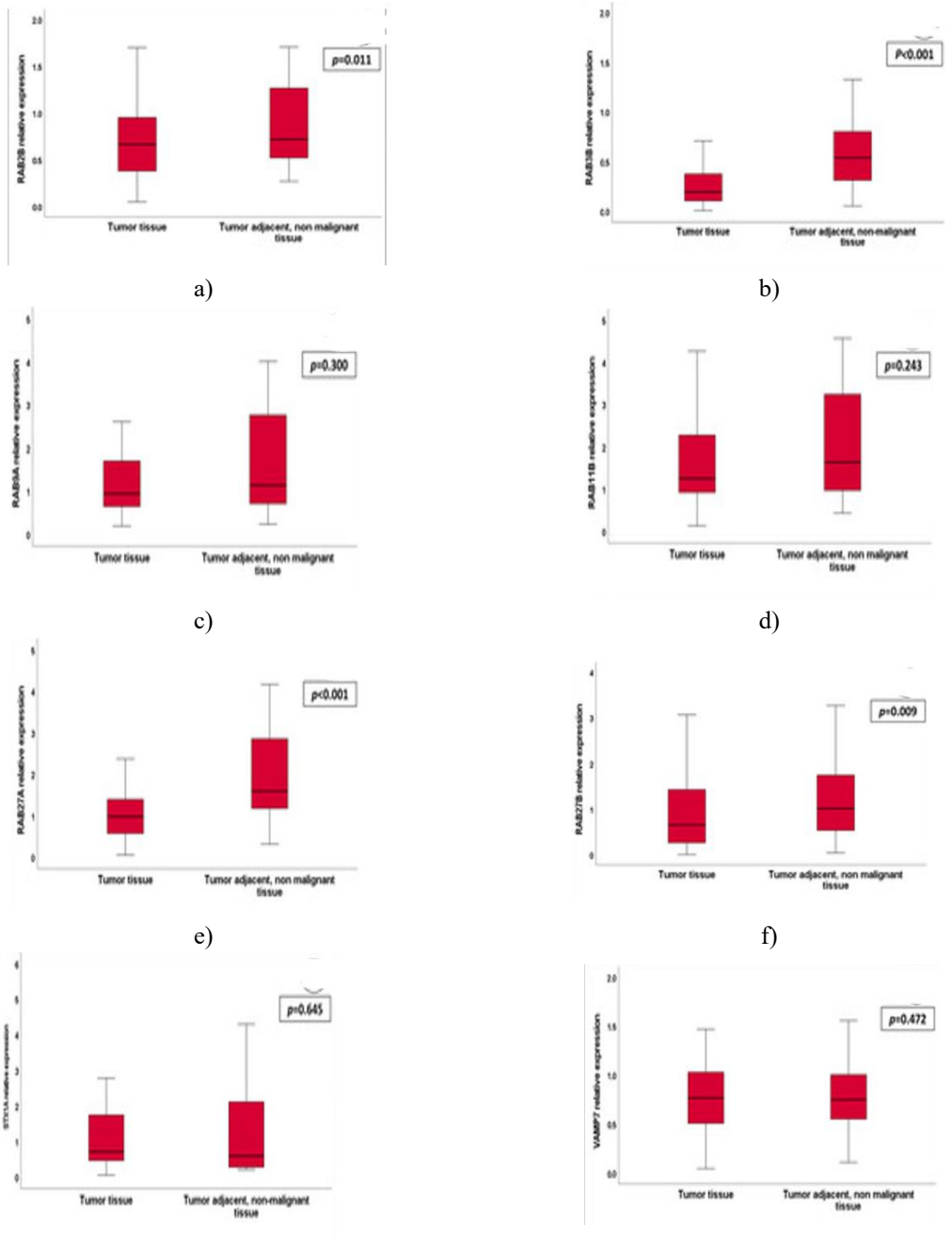


Figure 1. Relative expression comparisons for RAB2B (a), RAB3B (b), RAB9A (c), RAB11B (d), RAB27A (e), RAB27B (f), STX1A (g), and VAMP7 (h) in tumor (n = 109) versus adjacent normal tissue (n = 60). Decreases are evident for RAB2B, RAB3B, RAB27A, and RAB27B; the remaining genes show no significant deviation.

Gene expression patterns in relation to clinicopathological features

Assessment of clinical associations revealed that RAB27B expression correlated strongly with multiple parameters. Its expression progressively declined with advancing stage, ranging from the highest levels in in situ lesions to the lowest in stage IV cancers (p = 0.006; **Figure 2a**). A similar downward trend was evident across nodal categories, with N0 > N1 > N2 (p = 0.022; **Figure 2b**). Regarding grade, grade III tumors unexpectedly displayed higher RAB27B levels than grade I or II counterparts (p = 0.023; **Figure 2c**). Additionally, tumors

lacking distant spread had higher RAB27B expression compared with those demonstrating metastasis (p = 0.034; **Figure 2d**). No other gene showed significant associations with tumor stage, grade, or nodal involvement.

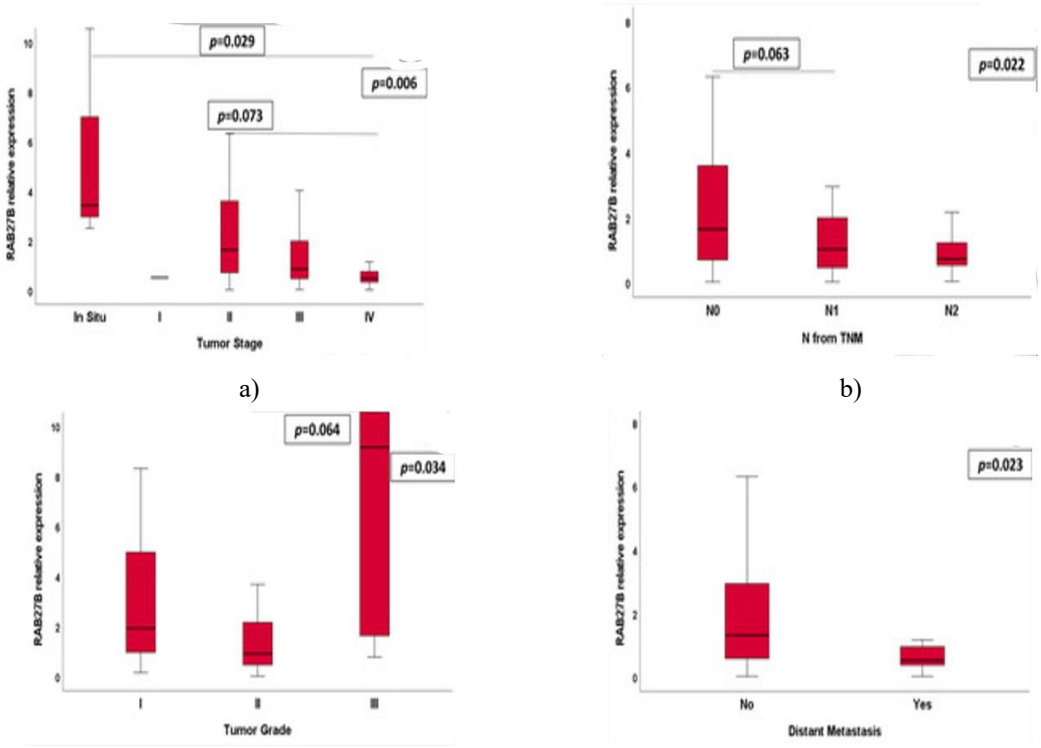


Figure 2. RAB27B expression across (a) tumor stage, (b) nodal status, (c) histologic grade, and (d) metastatic presence in CRC cases (n = 109). Lower RAB27B levels correspond to higher stage, lymph-node invasion, and distant spread; grade III tumors show comparatively elevated levels.

Among the remaining genes, RAB9A and RAB11B were linked specifically to distant spread, both showing reduced expression in metastatic cases (p = 0.008; p = 0.022; **Figures 3a and 3b**). Analysis according to TNM criteria showed that RAB2B varied significantly across T categories (p = 0.032; **Figure 4a**), whereas VAMP7 displayed a stepwise decrease with increasing T score (p = 0.036; **Figure 4b**). None of the other genes demonstrated notable associations with metastasis or T status.

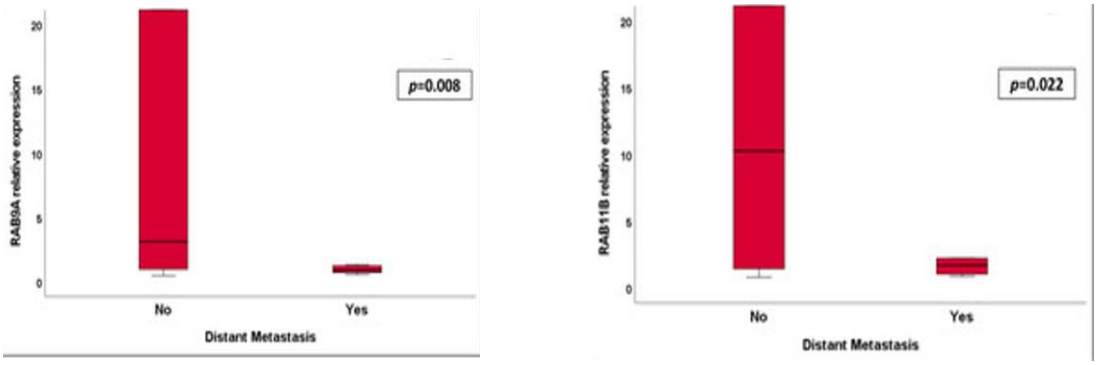
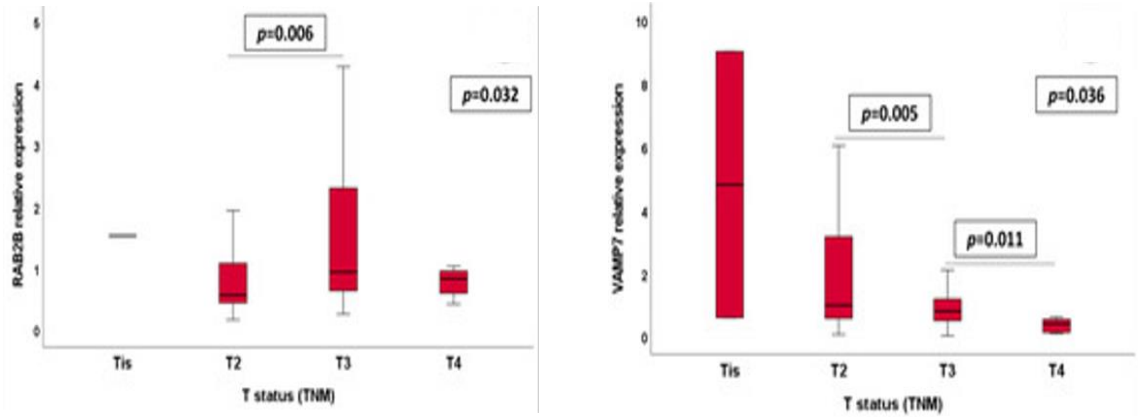


Figure 3. Expression of RAB9A (a) and RAB11B (b) in tumors with or without distant metastasis (n = 109), showing reduced levels in metastatic lesions.



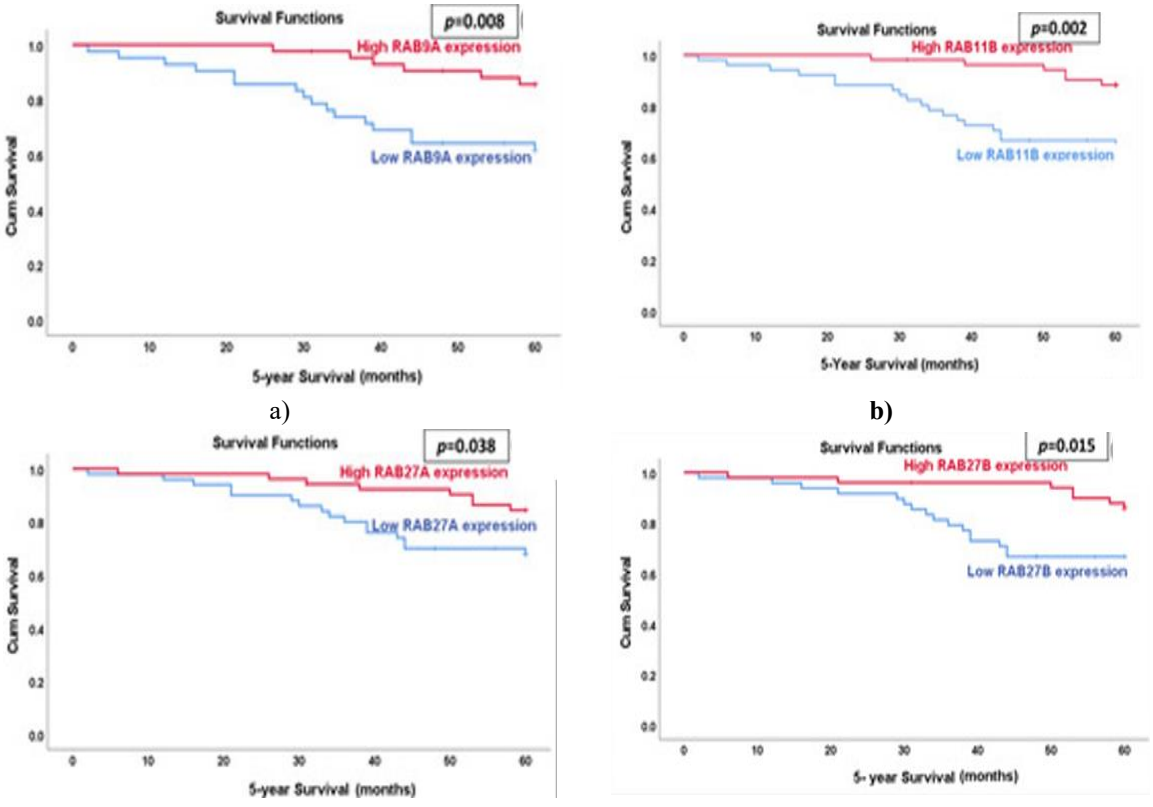
a) b)

Figure 4. RAB2B (a) and VAMP7 (b) expression across T categories (n = 109). RAB2B peaks in T3 versus T2, while VAMP7 diminishes progressively from T2 → T3 → T4.

Elevated expression predicts more favorable survival

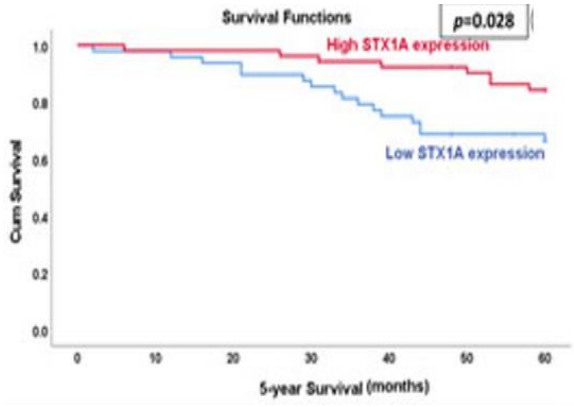
Survival analyses revealed that higher tumor expression of five of the eight investigated genes was linked to improved 5-year outcomes. Specifically, high levels of RAB27A, RAB27B, RAB9A, RAB11B, and STX1A were all associated with better overall survival at 5 years (p = 0.038; p = 0.015; p = 0.008; p = 0.002; p = 0.028; **Figure 5**).

The remaining genes did not show significant survival correlations.



a) **b)**

c) **d)**



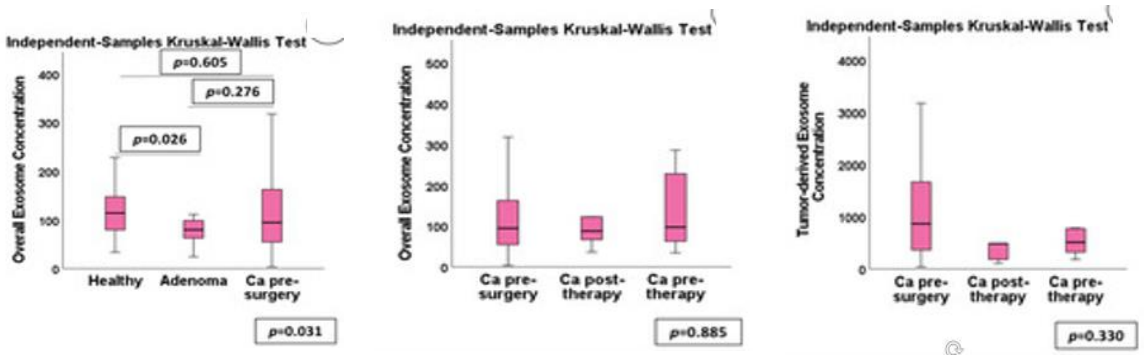
e)

Figure 5. Kaplan-Meier survival curves (n = 109) for RAB27A (a), RAB27B (b), RAB9A (c), RAB11B (d), and STX1A (e), demonstrating longer 5-year survival among patients with higher expression.

Quantification of total and tumor-specific plasma sEV levels

To extend our analysis, we measured circulating sEV concentrations across three groups: 65 colorectal cancer (CRC) patients sampled prior to surgery, 39 individuals with colorectal adenomas, and 39 healthy participants. A notable observation was that the adenoma group showed reduced total plasma sEV levels relative to healthy subjects (p = 0.026; **Figure 6a**).

In contrast, the comparisons involving CRC cases—either against healthy controls or against adenoma carriers—did not yield significant differences (p = 0.276 and p = 0.605).



a) b) c)

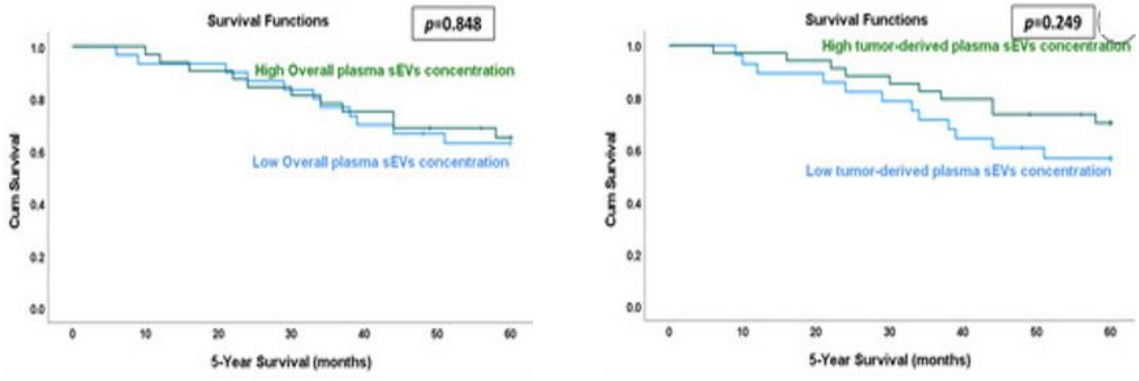
Figure 6. Overall plasma sEV measurements among healthy volunteers (n = 39), adenoma cases (n = 39), and CRC patients (n = 65) (a); and comparisons of total (b) and tumor-associated (c) plasma sEVs in CRC patients pre-surgery (n = 65), metastatic patients pre-therapy (n = 7), and metastatic patients after systemic treatment (n = 5).

We further contrasted both total and tumor-derived sEV concentrations in the 65 preoperative CRC cases with two metastatic subgroups: 7 patients sampled before systemic therapy and 5 sampled after systemic therapy. Neither the overall sEV levels nor the tumor-derived sEV levels differed significantly among these categories (p = 0.885 and p = 0.330; **Figures 6b and 6c**).

Association between plasma sEVs and survival

Using the median as the threshold, total plasma sEV concentrations measured before surgery in the CRC cohort showed no relationship with 5-year survival (p = 0.848; **Figure 7a**).

For tumor-specific sEVs, the pattern suggested a modest survival advantage for patients with higher levels, but the finding did not meet statistical significance (HR = 0.594; p = 0.249; **Figure 7b**).



a) b)

Figure 7. Kaplan-Meier survival plots for overall sEV levels (a) and tumor-derived sEV quantities (b) in CRC patients (n = 65). No statistically significant differences emerged, although elevated tumor-derived sEVs trended toward improved survival.

Correlations between sEV concentrations and gene expression

Correlation coefficients and p-values are summarized in **Table 3**.

A moderate positive association was found between total and tumor-derived plasma sEVs in preoperative CRC cases ($r = 0.408$, $p < 0.001$). Another moderate correlation appeared between RAB11B transcript levels and total sEVs ($r = 0.318$, $p = 0.045$). Strikingly strong correlations were detected among several gene pairs, the most pronounced being RAB2B with RAB27B and RAB9A with RAB11B ($r = 0.912$, $p < 0.001$; and $r = 0.812$, $p < 0.001$).

Table 3. Correlation statistics for sEV measurements and gene expression profiles.

Correlations	Overall Plasma sEVs Levels	Tumor-Derived Plasma sEVs Levels	RAB2B Expression	RAB3B Expression	RAB9A Expression	RAB11B Expression	RAB27A Expression	RAB27B Expression	VAMP7 Expression	STX1A Expression
Overall plasma sEVs levels										
Pearson Correlation	1	0.408 **	0.265	−0.100	0.291	0.318 *	0.041	−0.048	0.180	0.210
Sig. (2-tailed)		<0.001	0.103	0.541	0.069	0.045	0.800	0.770	0.267	0.213
Tumor-derived plasma sEVs levels										
Pearson Correlation	0.408 **	1	0.052	−0.059	0.047	0.019	−0.014	0.017	0.107	0.033
Sig. (2-tailed)	<0.001		0.753	0.719	0.772	0.905	0.931	0.918	0.511	0.848
RAB2B expression										
Pearson Correlation	0.265	0.052	1	0.483 **	0.799 **	0.456 **	0.438 **	0.231 *	0.229 *	0.537 **
Sig. (2-tailed)	0.103	0.753		<0.001	<0.001	<0.001	<0.001	0.026	0.026	<0.001
RAB3B expression										
Pearson Correlation	−0.100	−0.059	0.483 **	1	0.718 **	0.597 **	0.663 **	0.527 **	0.298 **	0.310 **
Sig. (2-tailed)	0.541	0.719	<0.001		<0.001	<0.001	<0.001	<0.001	0.004	0.002

RAB9A expression										
Pearson Correlation	0.291	0.047	0.799**	0.718**	1	0.812**	0.570**	0.294**	0.171	0.350**
Sig. (2-tailed)	0.069	0.772	<0.001	<0.001		<0.001	<0.001	0.006	0.114	<0.001
RAB11B expression										
Pearson Correlation	0.318*	0.019	0.456**	0.597**	0.812**	1	0.578**	0.126	0.159	0.615**
Sig. (2-tailed)	0.045	0.905	<0.001	<0.001	<0.001		<0.001	0.208	0.109	<0.001
RAB27A expression										
Pearson Correlation	0.041	−0.014	0.438**	0.663**	0.570**	0.578**	1	0.229*	0.226*	0.353**
Sig. (2-tailed)	0.800	0.931	<0.001	<0.001	<0.001	<0.001		0.021	0.022	<0.001
RAB27B expression										
Pearson Correlation	−0.048	0.017	0.231*	0.527**	0.294**	0.126	0.229*	1	0.397**	0.001
Sig. (2-tailed)	0.770	0.918	0.026	<0.001	0.006	0.208	0.021		<0.001	0.992
VAMP7 expression										
Pearson Correlation	0.180	0.107	0.229*	0.298**	0.171	0.159	0.226*	0.397**	1	0.223*
Sig. (2-tailed)	0.267	0.511	0.026	0.004	0.114	0.109	0.022	<0.001		0.027
STX1A expression										
Pearson Correlation	0.210	0.033	0.537**	0.310**	0.350**	0.615**	0.353**	0.001	0.223*	1
Sig. (2-tailed)	0.213	0.848	<0.001	0.002	<0.001	<0.001	<0.001	0.992	0.027	

** indicates significance at the 0.01 level (two-tailed); * indicates significance at the 0.05 level.

Previous publications have firmly established that sEVs participate in colorectal cancer biology at multiple stages, from early development to advanced progression [23]. Nevertheless, comparatively little information exists about the clinical implications of genes governing sEV biogenesis. For this reason, we investigated eight genes—RAB2B, RAB3B, RAB9A, RAB11B, RAB27A, RAB27B, STX1A, and VAMP7—and evaluated their relevance together with circulating total and tumor-derived sEV levels.

A particularly noteworthy observation involved RAB27B: tumor samples exhibited lower mRNA abundance than adjacent non-cancerous tissues. Moreover, RAB27B aligned with major clinical parameters—stage, nodal involvement, and metastatic status—with the highest expression occurring in early-stage tumors, in cases without lymph-node spread, and in those lacking distant metastasis. Elevated RAB27B also corresponded to better 5-year overall survival. These findings mirror outcomes from our accompanying bioinformatic assessments and agree with the report by Dong *et al*. showing reduced RAB27B expression in CRC and its association with favorable prognosis [24].

Divergent results do exist: Bao *et al*. described higher RAB27B expression in CRC relative to normal tissues and linked it to nodal spread, metastatic behavior, and worse survival [25]. Additional evidence from TCGA analyses by Hua *et al*. suggests RAB27B involvement in rectal adenocarcinoma metastasis [26], whereas Cheng *et al*. reported that RAB27B influences sEV secretion in CRC stem cells, promoting tumorigenic and immunosuppressive signaling [27].

Interestingly, we found that RAB27A mRNA levels were reduced in CRC tissue relative to adjacent non-tumorous areas, and higher expression appeared to be associated with a more favorable prognosis. Consistent with this pattern, Dong *et al*. also reported diminished RAB27A expression in CRC compared with benign tissue, and noted that loss of RAB27A protein correlated with distant spread, recurrence, and poorer survival [24]. In a related publication, Shi *et al*. likewise argued that elevated RAB27A levels predict better outcomes in CRC patients [28], although that same investigation paradoxically described higher RAB27A expression in cancerous tissue versus

non-cancer controls [28]. Moreover, our observations were supported by an independent evaluation using TCGA datasets. Although no significant link emerged between RAB27A transcript levels and plasma sEV concentrations, evidence still points to a critical involvement of RAB27A in sEV formation in CRC. For example, Huang and Feng demonstrated that RAB27A knockdown in hypoxic CRC cells suppresses exosome release and attenuates endothelial cell growth and migration [29]. Beyond exosome production, RAB27A may also influence CRC development by promoting cell stemness via NF-κB signaling [30], suggesting an sEV-independent mechanism through which it may shape clinical outcomes.

Another notable observation was that higher tumor expression of several assessed genes (RAB27A, RAB27B, RAB9A, RAB11B, and STX1A) corresponded to improved 5-year survival, in agreement with patterns highlighted in the TCGA-based analyses described in the "Gene selection" section. Elevation of these central sEV-biogenesis regulators could theoretically enhance overall sEV output [31]. In our dataset, this association with plasma sEVs was largely absent, apart from RAB11B, which showed a significant correlation with circulating sEV levels. Because plasma exosomes constitute a mixed population originating from various cell types—and considering that tumor-derived sEVs mainly exert localized effects, such as remodeling the extracellular matrix, dampening immune activity, and promoting cancer growth and dissemination [23]—the lack of correlation may simply indicate that tumor-released sEVs act predominantly within the tumor environment rather than generating major fluctuations in total plasma levels.

Within our cohort, individuals with adenomas exhibited lower circulating sEV concentrations compared with both healthy participants and CRC cases. Few published reports directly compare plasma sEV quantities among healthy subjects, adenoma carriers, and CRC patients. One study by Kobayashi *et al*. (2018) found that CRC patients displayed greater plasma sEV levels than healthy controls, hyperplastic polyps, or low-grade adenomas [32]. Their cohort included 5 adenocarcinoma cases (4 pTis, 1 pT1), 8 high-grade adenomas, 4 low-grade adenomas, 4 hyperplastic polyps, and 4 healthy individuals. However, the adequacy of their sample was limited, especially since the adenocarcinoma subgroup (n = 5) consisted primarily of non-invasive (Tis) tumors.

Despite the interesting findings, several constraints must be acknowledged. The sample size is a primary limitation. Additionally, a two-phase study design would have provided stronger validation, but it was not feasible due to the number of enrolled participants. Our cohort also had uneven representation of stage I and stage IV CRC cases, and age variation across subgroups may have introduced confounding. Lastly, for exosome isolation and quantification, we relied solely on assays targeting CD9, which restricts the characterization of plasma-derived vesicles.

Conclusion

Taken together, our results indicate that the exosome-generation machinery may hold biological and prognostic importance in CRC, with RAB27A, RAB27B, RAB9A, RAB11B, and STX1A emerging as promising markers for survival prediction. Although we did not detect a statistically significant relationship between these genes and total plasma sEV levels, the data suggest that their influence may be more confined to tumor-originating sEVs. Additional research is required to clarify these relationships and determine their possible clinical applications.

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