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Diagnostic and Prognostic Potential of Long Non-Coding RNAs GAS5, MALAT1, CCAT2, HOTAIR and H19 in Colorectal Cancer Cases with Peritoneal Metastases

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HIGHLIGHTS

- Colorectal cancer (CRC) is one of the most important reasons of cancer deaths in all over the world.
- Long non-coding RNAs (lncRNAs) have regulatory effects in regulating tumor process of CRC.
- We studied lncRNAs GAS5, HOTAIR, CCAT2, MALAT1 and H19 in metastatic CRC cases.
- All these lncRNAs may be used as diagnostic markers in peritoneal metastatic CRCs.

Abstract: It was aimed to evaluate long non-coding RNAs (lncRNAs) in terms of diagnosis and prognosis in colorectal cancer (CRC) cases with peritoneal metastasis who underwent cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. Paraffinized 32 CRC tissue and 32 adjacent normal colon tissue of 32 cases, and paraffinized metastatic 10 peritoneal tumor tissue and 10 adjacent normal peritoneal tissues of 10 cases were included in. Expression levels of lncRNAs GAS5, MALAT1, CCAT2, HOTAIR and H19 were measured by qRT-PCR, clinicopathological and demographic characteristics of the patients were also recorded. 11 (34%) patients were female and 21 (66%) were male. The mean age was 54.59±11.97 (24-73 years). Expression levels of GAS5 decreased significantly in colon tumor tissues compared to normal colon tissues ($p<0.001$), while HOTAIR, CCAT2, MALAT1 and H19 levels were increased significantly ($p<0.001$). In tissues where the tumor has metastasized to the peritoneum, the expression levels of GAS5 decreased significantly compared to the adjacent normal peritoneal tissue ($p=0.028$), while HOTAIR, CCAT2, MALAT1 and H19 levels were increased significantly ($p=0.007$, $p=0.005$, $p=0.009$, $p=0.028$, respectively). No

association was found between lncRNAs and survival. In conclusion, lncRNAs GAS5, HOTAIR, CCAT2, MALAT1 and H19 may be used as diagnostic markers in CRC cases with peritoneal metastasis.

Keywords: Colorectal cancer; long non-coding RNA; hyperthermic intraperitoneal chemotherapy; peritoneum; metastasis.

INTRODUCTION

Colorectal cancer (CRC) is one of the eminent cause of cancer deaths in all over the world and is one of the most common cancers in both men and women. The risk of CRC developing is affected by both environmental and genetic factors. Actually, most CRCs are sporadic rather than familial [1,2]. The most important prognostic factors in colon cancers are stage of disease and presence of metastases [1,2]. In patients with colon cancer, peritoneal metastasis (PM) is found in nearly 10-15% of cases. However, PM has a worse prognosis in cases with other metastasis sites. Clinical trials and meta-analyses have provided clear evidence that cytoreductive surgery (SRC) plus hyperthermic intraperitoneal chemotherapy (HIPEC) can clearly ameliorate overall survival in comparison to systemic chemotherapy alone in selected CRC patients with PM. In general, it is recommended that CRC cases with PM should be managed by a multidisciplinary team in experienced centers and also SRC plus HIPEC should be considered for selected individuals [3]. On the other hand, tumor biology of colorectal cancer, metastasis dynamics and possible biomarkers that may indicate the course of the disease are intensively investigated [1,3].

Long non-coding RNAs (lncRNAs) are generally existing in nucleus and cell cytoplasm. They are bigger than 200 nucleotides, and as a crucial information, lncRNAs do not encode proteins [4]. Comparison to small RNAs, their sequences are longer and structure is more complex. Additionally, their various mechanisms are involved in the regulation of gene expression. LncRNAs have important roles in the biological processes of cells. Moreover, they play pivotal roles in emergence and development of many diseases [4]. Various published papers have exhibited that lncRNAs can induce cancer development and can also affect tumor biology. In other words, lncRNAs play essential roles in molecular signaling pathways, cell cycle, cell apoptosis and autophagy, tumor growing and behavior, angiogenesis, immune system responses, and a dynamic process called as epithelial-mesenchymal transition [5].

In the literature, there are insufficient and sometimes contradictory data on the role of lncRNAs in tumor behavior in patients with colon cancer and especially CRC cases with PM. There is a need to reveal biomarkers that are effective on the diagnosis and course of the disease in CRC.

In this study, it was aimed to evaluate five lncRNAs (GAS5, HOTAIR, CCAT2, MALAT1 and H19) in terms of diagnosis and prognosis in CRC cases with peritoneal metastasis who underwent cytoreductive surgery and HIPEC. Additionally, it was targeted to investigate whether these lncRNAs may use as biomarkers for CRC cases with PM.

MATERIAL AND METHODS

Obtaining Colon and Peritoneal Tissues

First of all, ethics committee approval was obtained from the University of Health Sciences, Kartal City Hospital (2020/10/374). Formalin-Fixed Paraffin-Embedded (FFPE) colon tumor tissues and corresponding normal tissues of 32 CRC patients aged between 18-75 years, who underwent cytoreductive surgery and HIPEC at Kartal Kosuyolu Training and Research Hospital between 2018-2021 were included in the study. Additionally, paraffinized 10 metastatic peritoneal tumor tissues (CRC metastasis) and 10 normal peritoneal tissues adjacent to the metastatic peritoneal tumor were included in the research. Cytoreductive surgery and HIPEC procedure were performed by the same surgical team and anesthetists. Moreover, all patients were given same intraperitoneal (IP) chemotherapy agents in HIPEC procedure (5-Fluorouracil+leucovorin+oxaliplatin). Patients under 18 years of age, patients who used different IP chemotherapy agents and cases with another concomitant tumors were excluded from the study.

RNA Isolation from FFPE

Paraffin sections of 10-35 µm thickness were placed in a suitable tube. Total RNA was extracted from FFPE tissues using “Lucigen MasterPure™ Complete DNA and RNA Purification Kit” (Lucigen, Middleton, WI, USA) according to the manufacturer’s instructions. RNA concentration and purity were evaluated by Take3 Micro-Volume Plate - BioTek Instruments (Agilent, USA) spectrophotometers.

cDNA Synthesis and Quantitative Real Time Polymerase Chain Reaction

Total RNA samples of 500 ng/ul were used for cDNA reverse transcription using 'Transcriptor First-Strand cDNA Synthesis Kit' (Roche, Switzerland). The used primers were given in Table 1. In order to determine lncRNA expression levels, SYBR Green Master Mix (Applied Biosystems) was used according to the protocol for expression levels of GAS5, HOTAIR, CCAT2, MALAT1 and H19 were evaluated. After adding the relevant mixtures, qRT-PCR was performed in the LightCycler® 480 Instrument II device and. LncRNAs expression levels were tested using delta delta CT method. GAPDH was used as an internal control.

Table 1. List of primers that used in the study

Genes	Primer Sequences 5'---> 3'	Annealing Temperature ^{0}C
MALAT1-F	GAAGGAAGGAGCGCTAACGA	61
MALAT1-R	TACCAACCACTCGCTTTCCC	
CCAT2-F	CTCTGGAGCCATACGTGACA	59
CCAT2-R	TCAGGTCACTTGTGCCGTT	
HOTAIR-F	GGGTGTTGGTCTGTGGAACT	59
HOTAIR-R	CAGTGG-GGAACTCTGACTCG	
GAS5-F	CTTGCCTGGACCAGCTTAAT	59
GAS5-R	CAAGCCGACTCTCCATACCT	
H19-F	TCAGCTCTGGGATGATGTGGT	62
H19-R	CTCAGGAATCGGCTCTGGAAG	
GAPDH-F	TGCACCACCAACTCCT	55
GAPDH-R	GACGCAGGGATCATGT	

Statistical analysis

All statistical analyzes were accomplished with SPSS version 20.0 (SPSS Inc., IBM Corp). The compatibility with the normal distribution was evaluated with the Kolmogorov-Smirnov test. Student's t test was utilized for data with normal distribution, and non-parametric Mann-Whitney U test was used to check against quantitative variables in case of non-normal distribution. Chi-square test was applied for qualitative variables among study groups. Survival was tested using the Kaplan-Meier method. A 'p value' <0.05 was considered as statistically significant.

RESULTS

Of the patients whose paraffinized tumor tissue were studied in the research, 11 (34%) were female and 21 (66%) were male. The mean age was 54.59±11.97 (24-73 years). Diabetes mellitus was present in 5 patients (15%), essential hypertension in 8 patients (25%), coronary artery disease in 3 patients (0.9%). Again, 5 of the patients had a history of smoking (15%). Twenty patients (62%) had a history of previous surgery (right/left hemicolectomy, low anterior resection, appendectomy, etc.) for the disease. Ten of the cases were of normal weight (31%), 13 of them were overweight (41%), and 9 of the patients were obese (28%). Two of them had a history of previous radiotherapy, and 18 of them had a history of previous chemotherapy. The mean disease severity score was 5±3.2, and the mean preoperative PCI score was 2±1.4 (Table 2).

Table 2. Demographic and clinical characteristics of the patients

Demographic features	Number of cases/rate
Age (mean±SD)	54.59±11.97
Sex	
Female	11 (34%)
Male	21 (66%)
Body mass index	
Normal	10 (31%)
Overweight	13 (41%)
Obese 1	7 (22%)
Obese 2	2 (0.6%)
Comorbid conditions	
Diabetes	5 (15%)
Hypertension	8 (25%)
Coronary heart disease	3 (0.9%)
Tumor cite	
Colon	26 (81%)
Rectum	6 (19%)
Smoking	5 (15%)
Previously operation anamnesis	20 (62%)
Previously chemotherapy anamnesis	18 (56%)
Previously radiotherapy anamnesis	2 (0.6%)
Illness severity score (mean±SD)	5±3.2
Peritoneal cancer index (mean±SD)	2±1.4
Sitoreductive surgery process	
Peritonectomy	16 (50%)
Cholecystectomy	8 (25%)
Splenectomy	6 (19%)
Small bowel resection	10 (31%)
Total colectomy	3 (0.9%)
Survival time (months) (mean±SD)	21.48±9.16
Survival/exitus	18/14 (56%/44%)
Relapse duration (months) (mean±SD)	10.38±5.01

All patients underwent cytoreductive surgery and the same HIPEC chemotherapy regimen (5-Fluorouracil+leucovorin+oxaliplatin) was applied to all patients. The median preoperative carcinoembryonic antigen (CEA) level was 4.55 mg/dl (0.8-57.20), and the median CEA level was 3.3 mg/dl (1.9-17) at 6 months postoperatively. Median preoperative CA 19-9 level was 11.3 mg/dl (0.8-230), and median CA19-9 level was 12.7 mg/dl (0.8-189) at 6 months postoperatively. Hepatotoxicity developed in 2 (6%) patients, and nephrotoxicity developed in 7 (22%) patients. When the survival analysis was performed, it was determined

that the mean survival after cytoreductive surgery+HIPEC was 21.48±9.16 months, and the survival decreased to 47.5% at the end of the 32nd month.

There was no significant relationship between long non-coding RNA expression levels and survival. Survival at the end of the 10th month was 90% for people with a normal body mass index (BMI), while this rate was 54.1% for those with a normal weight. In the obese 1 (BMI=30-35) category, the survival rate was 25.7% at the end of the 6th month and 0% at the end of the 2nd month in the obese 2 category (BMI=35-40). As a result of the log rank test, the difference between the categories was found to be significant at the 95% confidence level (X2=9.301; p=0.026). Kaplan-Meier survival charts are shown in Figure 1. Pearson correlation analysis between lncRNAs, preoperative tumor markers levels and survival status were shown in Table 3.

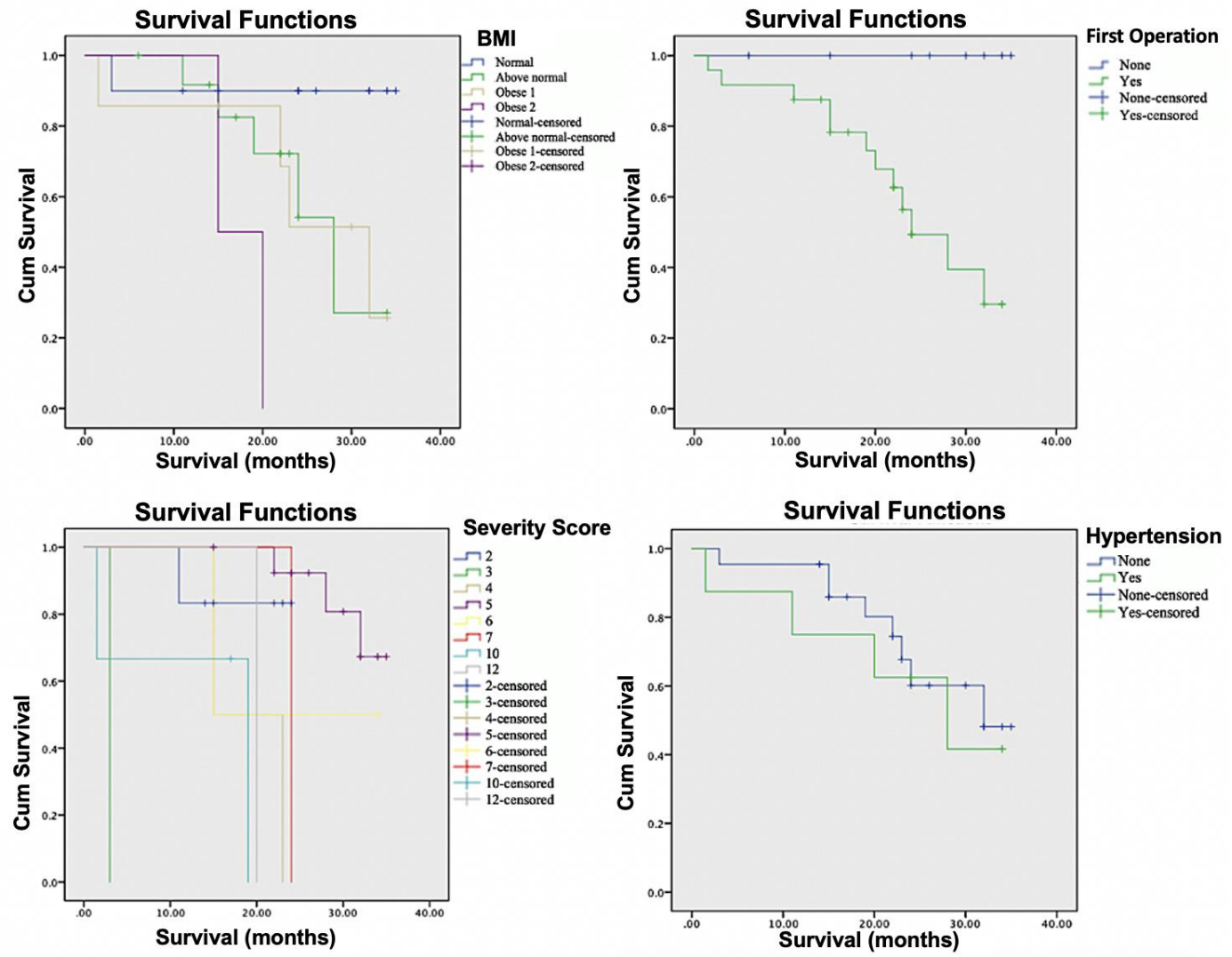

Figure 1. Kaplan-Meier survival charts of colorectal cancer patients in view of body mass index (BMI), first operation (previously operation anamnesis), severity index score and presence of hypertension

Table 3. Pearson correlation analysis between lncRNAs, preoperative tumor markers levels and survival status

		GAS5 CT	MALAT1 CT	CCAT2 CT	H19 CT	HOTAIR CT	Preoperative Ca 19-9	Preoperative CEA	MALAT1 PT	GAS5 PT	CCAT2 PT	H19 PT	HOTAIR PT	Survival/ex
GAS5 CT	Pearson	1	.417*	.229	.361*	.376*	.052	-.100	-.303	-.307	-.127	-.132	-.042	.096
	P value		.018	.208	.042	.034	.779	.586	.395	.388	.727	.734	.908	.615
MALAT1 CT	Pearson	.417*	1	.329	.519**	.583**	.185	.214	-.235	-.196	.044	.434	.499	.212
	P value	.018		.066	.002	.000	.310	.239	.514	.588	.905	.243	.142	.261
CCAT2 CT	Pearson	.229	.329	1	.416*	.469**	.194	.155	-.218	.077	.636*	-.293	-.066	.049
	P value	.208	.066		.018	.007	.288	.396	.545	.832	.048	.444	.856	.799
H19 CT	Pearson	.361*	.519**	.416*	1	.589**	.085	-.121	.071	-.026	-.095	.441	.309	.082
	P value	.042	.002	.018		.000	.643	.510	.844	.944	.794	.235	.385	.667
HOTAIR CT	Pearson	.376*	.583**	.469**	.589**	1	-.030	-.072	-.289	-.495	-.579	.127	-.272	.159
	P value	.034	.000	.007	.000		.869	.693	.418	.146	.080	.744	.447	.402
Preoperative Ca 19-9	Pearson	.052	.185	.194	.085	-.030	1	.200	.140	.319	.692*	-.179	.187	,456*
	P value	.779	.310	.288	.643	.869		.273	.700	.368	.027	.645	.605	.011
Preoperative CEA	Pearson	-.100	.214	.155	-.121	-.072	.200	1	.055	.178	.375	-.105	-.110	-.063
	P value	.586	.239	.396	.510	.693	.273		.880	.623	.286	.789	.762	.739
MALAT1 PT	Pearson	-.303	-.235	-.218	.071	-.289	.140	.055	1	.886**	.330	.326	.592	.267
	P value	.395	.514	.545	.844	.418	.700	.880		.001	.352	.392	.071	.522
GAS5 PT	Pearson	-.307	-.196	.077	-.026	-.495	.319	.178	.886**	1	.630	.237	.613	.061
	P value	.388	.588	.832	.944	.146	.368	.623	.001		.051	.539	.060	.885
CCAT2 PT	Pearson	-.127	.044	.636*	-.095	-.579	.692*	.375	.330	.630	1	.215	.470	.364
	P value	.727	.905	.048	.794	.080	.027	.286	.352	.051		.579	.171	.375
H19 PT	Pearson	-.132	.434	-.293	.441	.127	-.179	-.105	.326	.237	.215	1	.640	.788*
	P value	.734	.243	.444	.235	.744	.645	.789	.392	.539	.579		.063	.035
HOTAIR PT	Pearson	-.042	.499	-.066	.309	-.272	.187	-.110	.592	.613	.470	.640	1	.567
	P value	.908	.142	.856	.385	.447	.605	.762	.071	.060	.171	.063		.143
Survival/ex	Pearson	.096	.212	.049	.082	.159	.456*	-.063	.267	.061	.364	.788*	.567	1
	P value	.615	.261	.799	.667	.402	.011	.739	.522	.885	.375	.035	.143	

CT: Colon tumor PT:Peritoneal tumor Ex: Exitus CEA: Carcinoembriyonic antigen

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Based on the history of previous surgery for the disease; while survival rate was 100% in those who did not have a previous operation, survival decreased to 29.6% at the end of the 21st month in those with a history of previous operation. As a result of the log rank test, the difference between the categories was found to be significant at the 95% confidence level (X2=5.882; p=0.015).

According to the results, it was determined that GAS5 expression levels in colon tumor tissue showed a significant decrease compared to normal colon tissue adjacent to the tumor (p<0.001). HOTAIR, CCAT2, MALAT1 and H19 expression levels were significantly increased in tissues with colon tumors compared to normal colon tissues adjacent to the tumor (p<0.001 for each parameter). In tissues where colon tumor metastasized to the peritoneum, GAS5 expression levels were significantly decreased compared to the adjacent normal peritoneal tissue (p=0.028). In tissues where colon tumor metastasized to the peritoneum, HOTAIR, CCAT2, MALAT1 and H19 expression levels were significantly increased compared to the adjacent normal peritoneal tissue (p=0.007, p=0.005, p=0.009, p=0.028, respectively). Relative expression levels of lncRNAs in normal vs tumor tissues are shown in Figure 2.

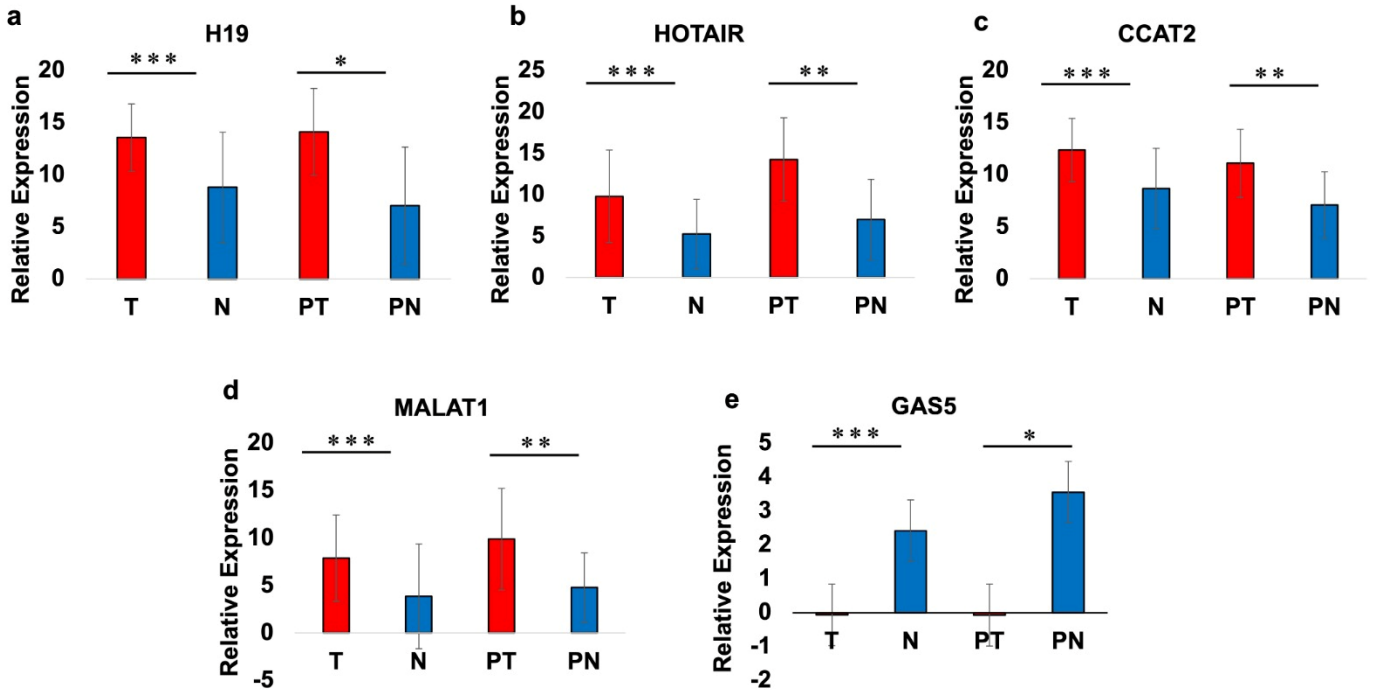

Figure 2. Relative expression levels of long non-coding RNA's in normal colon tissues vs colon tumor tissues, normal peritoneum tissues vs peritoneal metastatic cancer tissues (T:Colon tumor tissue, N:Colon normal tissue, PT: Peritoneal metastasis, PN: Peritoneal normal tissue, **:p<0.05, **:p<0.01, ***:p<:0.001*)

DISCUSSION

To the best of our knowledge, this is the first study that evaluate long non-coding RNAs in terms of diagnosis and prognosis in colorectal cancer (CRC) patients with peritoneal metastasis who underwent cytoreductive surgery (CS) and hyperthermic intraperitoneal chemotherapy (HIPEC).

CRC is still a kind of commonly seen carcinoma with high mortality rates. Metastasis develops in peritoneal cavity in nearly 10% of CRC cases at the time of diagnosis. On the other hand, metastasis occurs in 40% of CRC patients during clinical follow-up [6]. Without treatment, the 5-year overall survival of individuals with stage IV CRC is unfortunately not long and the average survival is 6 months. In recent years, cytoreductive surgery plus HIPEC has proven to be an efficient treatment for CRC with peritoneal metastases [7]. In particular, the administration of 5-FU+leucovorin±oxaliplatin (FOLFOX)-based systemic chemotherapy or irinotecan (FOLFIRI) improved overall survival up to 20 months. In the literature, it has been shown that cytoreductive surgery plus HIPEC can significantly ameliorate the survival of these cases compared to systematic chemotherapy alone in patients with stage IV CRC. In line with this promising scientific evidence, understanding the tumor and metastasis biology in more detail in CRC cases with peritoneal metastasis, finding more successful biomarkers in terms of predicting survival, and personalizing the treatment for patients as much as possible will increase the success of treatment and prolong patient survival at a much more satisfactory level [8,9].

Recently, it has been exhibited that non-coding RNAs play a pivotal role in many types of malignities and also their progression [10]. It is a well-described fact today that ncRNAs play inevitable roles in a variety of cellular and physiological functions. Long non-coding RNAs participate in many kinds of cellular processes such as cell proliferation, differentiation, apoptosis, and metastasis, and interacts with miRNAs [11]. Moreover, lncRNAs can also influence the chromatin structure and lead to modulation of gene expression [12]. These essential steps can explain why lncRNAs are very important for CRC development and its metastasis.

First, in 2014, Yin and coauthors [13] investigated the role of GAS5 in colorectal cancer. They used real-time PCR to investigate the expression levels of GAS5 in tumor tissues from 66 patients with CRC and adjacent normal colon tissues. At the end of the study, they reported that low expression of GAS5 was closely associated with bigger tumor size and advanced TNM stage. They suggested that GAS5 could serve as a candidate diagnostic biomarker in human CRC. In 2016, Kong and coauthors [14] used 15 lncRNAs in 51 stage IV CRC and 57 stage I/II CRC with liver metastases in order to investigate the diagnostic and prognostic value of lncRNAs on hepatic metastases in CRC cases. Finally, they found that expression levels of four lncRNAs (Yiya, GAS5, H19 and MEG3) were significantly different between CRC cases with liver metastases and without. They also showed that altered expression levels of GAS5 or Yiya associated with the poor prognosis of patients with early-stage CRC. In the same year, Zheng and coauthors [15] revealed that the long non-coding RNA GAS5 contributes to lymphatic metastasis in CRC [15]. In this thesis, we found that GAS5 expression levels were significantly lower in colon tumor tissues compared to adjacent normal colon tissue, in line with the literature. In addition, GAS5 expression was expressed in tumor tissues metastasized to the peritoneal tissue and adjacent normal peritoneal tissues of CRC cancer patients who underwent cytoreductive surgery and HIPEC for the first time in the literature.

In 2014, Ji and coauthors [16] examined the relationship between colorectal cancer and MALAT1 in vivo and in vitro. At the end of the study, they suggested that overexpression of MALAT1 could promote cell proliferation and tumor migration in vitro and promote tumor growth and metastasis in mice. In the same year, Zheng and coauthors [17] accomplished a study investigating the role of MALAT1 in the prognosis of stage II/III CRC patients. In their study, they found that MALAT1 expression levels increased significantly in patients with CRC and suggested that high expression levels of MALAT1 in CRC tissues (especially in stage II/III CRC patients) could be used as a negative prognostic marker. In another recent study, Liu and coauthors [18] reported that MALAT1 and HOTAIR expression levels were significantly increased in CRC cases with liver metastases and suggested that high HOTAIR/MALAT1 ratio might be a prognostic signature for liver metastasis and overall poor survival. In accordance with the published studies with our thesis team, we determined that MALAT1 expression levels were significantly prominent in colon cancer tissues compared to adjacent normal tissue. Moreover, we examined the MALAT1 expression levels in metastasized and normal peritoneal tissues of patients with CRC who underwent cytoreductive surgery and HIPEC for the first time in the literature, and a significant increase was observed, but no correlation was found between expression levels and prognosis.

Ozawa and coauthors [19] looked at the expression levels of CCAT2, another long non-coding RNA, in colon tumor tissues and adjacent normal tissue in their study in 2017 and reported that CCAT2 increased significantly in tumor tissue. They stated that high expression levels of CCAT2 were significantly associated with survival and could be used as a biomarker. In another study, Wang and coauthors [20] looked at CCAT2 expression levels in colon cancer tissues and adjacent normal tissue and found that CCAT2 levels increased significantly. In this thesis study, we revealed that CCAT2 expression levels increased significantly in tumor tissues in accordance with the literature, and for the first time in the literature, we found that CCAT2 expression levels were significantly increased in tumor tissues metastasizing to the peritoneum compared to normal peritoneal tissue.

To analyze the relationship between long noncoding RNA levels and CRC prognosis, Li and coauthors [21] conducted a study that detected H19 and 20 other long noncoding RNA expression levels in 30 CRC patients and included a 120-week follow-up. As a result, they showed that H19 expression levels were significantly increased in tumor tissues. They stated that there was no significant association between the age, sex, tumor size or TNM stage of these patients and their H19 levels. In our study, H19 expression levels were found to be significantly higher in colon tumor tissues compared to normal colon tissues, which is consistent with this study. On the other hand, for the first time in the literature, we found that H19 expression levels were higher in CRC metastasis tumor tissue in the peritoneum compared to the adjacent normal peritoneal tissue, and it was determined that H19 expression levels in peritoneal tumor tissue were associated with survival. Zhao and coauthors [22] found that H19 and MALAT1 expression levels in tumor tissues of CRC cases with type 2 diabetes were significantly higher than in normal colon tissues. Zhang and coauthors,

on the other hand, in a recent study [23] showed that H19 expression levels were significantly increased in primary tumor and metastatic tissues, associated with poor prognosis in CRC. They suggested that H19 could be a potential biomarker for predicting prognosis as well as a therapeutic strategy for CRC.

In a study by Kogo and coauthors [24] in 2011, they examined the expression and function of HOTAIR in patients with stage IV CRC with liver metastases and poor prognosis. At the end of the study, they suggested that HOTAIR expression levels were higher in colon cancerous tissues than in adjacent non-cancerous tissues, and that high HOTAIR expression was closely associated with the presence of liver metastases. In addition, the authors stated that the prognosis of patients with high HOTAIR expression levels is relatively worse. On the other hand, Peng and coauthors, in their studies evaluating colon tumor and adjacent normal colon tissues, showed that HOTAIR expression levels increased significantly in distant metastasis and colon cancer tissues [25]. In our study, we determined that HOTAIR expression levels were significantly higher in colon tumor tissues compared to adjacent normal tissue, which was consistent with the literature. In addition, for the first time in the literature, patients with CRC who underwent cytoreductive surgery and HIPEC were reported.

This study has some limitations. First, follow-up duration of CRC patients is not extremely long which may affect the relationship between long non-coding RNA and survival. On the other hand, study population was relatively small. Moreover, this current preliminary study is a single centered study that may also influence the survival rates.

In conclusion, in line with the results of this current pioneering study, we believe that all these lncRNAs may be used as diagnostic markers because their expression levels change significantly, their expression levels are correlated with each other, and there are also significant expression changes in metastatic tumor tissues detected in the peritoneum.

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Conflicts of Interest: The authors declare no conflict of interest.

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