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CLINICAL STUDY

Predictive value of KRAS/NRAS, IL-4 VNTR and HPV alterations in metastases of colorectal cancer

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ABSTRACT

PURPOSE: The main aim of the study was to evaluate the potential roles of KRAS/NRAS proto-oncogenes, IL-4 VNTR variants and HPV prevalence in colorectal cancer metastasis. As the second aim, the interactions of the analyzed genes and viral sequences with both clinicopathological variables and each other were targeted.
METHODS: DNA was extracted using AmoyDx FFPE DNA Extraction kit from paraffin-embedded colorectal tumor tissue samples (n = 60). NRAS/KRAS mutational profiles were determined with real-time polymerase chain reaction using AmoyDx KRAS/NRAS Mutation Detection Kit. Genotyping of IL-4 VNTR was made with PCR. HPV detection was analyzed by PCR with both GP5+/GP6+ consensus primers and type-specific primers for HPV-16 and HPV-18. SPSS v22 (IBM) statistics software was used for all statistical analyses.
RESULTS: From the demographical/clinicopathological parameters, age and biopsy specimens revealed an association with metastasis. KRAS mutation rate was as high as 65 % in the patients and the most prevalent mutation type was G12D. Metastasis risk was 3.19-fold increased in KRAS-mutated patients compared to KRAS-negative ones. IL-4 VNTR genotypes/alleles were not associated with metastasis in our analysis. The frequency of HPVs in our colorectal cancer cohort was 36.7 %, but HPV positivity was not found to be associated with metastasis. A significant association was found between HPV and NRAS mutations; NRAS wild-type status acted as a protective factor by 7.5-fold for HPV negativity.
CONCLUSION: Our study comprehensively and concomitantly evaluated several potential molecular risk factors. Future studies designed in such combined approaches will substantially contribute to better manage colorectal cancer tumorigenesis from molecular biological perspective *(Tab. 6, Fig. 2, Ref. 40)*. Text in PDF *www.elis.sk.*

KEY WORDS: colorectal cancer, KRAS, NRAS, IL-4, human papillomavirus, metastasis.

Introduction

Colorectal cancer (CRC) is one of the leading reasons of cancer death among both men and women in Western World and the risk is increased with the effects of external factors such as consumption of alcohol, red meat, high fat and low fiber food and sedentary lifestyle (1–2). CRC comprises nearly 11 % of all diagnosed cancer types worldwide and approximately 1.800.000 new cases occur annually (3–4). Early cancer diagnosis is an important step to qualify patients for surgical resection which is restricted with small percentage of patients and only a palliative approach in case of metastases. Hence, both early diagnosis and systemic treatment involving chemotherapy and molecularly targeted therapies are significant diagnostic and treatment strategies in the management of CRC (5).

Excluding external factors related with lifestyle briefly mentioned above, genetic factors also play substantial role in colon carcinogenesis. From the molecular perspective, the current and accepted model of colon carcinogenesis is characterized by a progressive multistep accumulation of genetic, chromosomal and epigenetic alterations, arising from a crypt lesion from small adenomatous polyps to malignant carcinoma (1, 6). Genetic background of colorectal tumorigenesis is driven and shaped by key signaling pathways such as the mitogen-activated protein kinase MAPK (RAS-RAF-MEK-ERK) and the phosphoinositide 3-kinase (PI3K). MAPK pathway regulates cell growth, differentiation, proliferation, apoptosis via stimulating the epidermal growth factor receptor (EGFR) and leading to the activation of a cascade of phosphorylation events (3). RAS members of proto-oncogenes (KRAS, HRAS, and NRAS genes) in the RAS/RAF/MAPK pathway are appropriate candidates for an effective targeted therapy and the enzymes encoded by the three RAS family genes share a

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high protein homology over 90 % (2). KRAS, NRAS, and HRAS activating mutations account for about 85 %, 15 % and for less than 1 % of all RAS mutations in human tumors, respectively. KRAS gene encodes for a guanosine triphosphate/guanosine diphosphate binding protein down-stream of the EGFR (7–8). Testing for 'hotspots' mutations in KRAS codons 12 and 13 (exon 2) is a routinely applied clinical practice for patients with advanced colorectal cancer prior to treatment with anti-EGFR monoclonal antibodies such as cetuximab. The mutations in these codons are associated with a lack of response to systemic approaches such as chemotherapy (2). Although the major checkpoints in KRAS gene to identify patients who can benefit from anti-EGFR therapy are majorly codons 12/13, some CRC patients with wild-type KRAS codons 12/13 also cannot take advantage of anti-EGFR therapy. Hence, this situation necessitates additional rare mutation screening at other locations of KRAS and other genes such as NRAS (5, 7).

Interleukin-4 (IL-4) plays a significant role in the control of cell growth and the regulation of the immune system and especially in Janus kinase family (JAK)-Signal Transducer and Activator of Transcription (STAT) signaling pathway in tumorigenesis. In the analysis of HT-29 and WiDr colon cancer cell lines, it was observed that tyrosine phosphorylation of JAK1, JAK2, Tyk2, IRS-1 and STAT-6 protein was induced by IL-4 (9). Thereafter, Chang et al (10) for the first time demonstrated that IL-4 in a dose-dependent manner could inhibit cell growth of colon carcinoma cell lines (HT29 and WiDr) via activating Stat1. On the other hand, the role of IL-4 on tumorigenesis seems to be a bit contradictory, dependent upon tumor type and needs to be further evaluated. Todaro et al (11) reported that stem cell marker $CD133^{+}$ cells could produce IL-4 to avoid apoptosis and promote cancer cell survival via upregulation of antiapoptotic genes. IL-4 gene located on 5q31.1 is in a cluster of cytokine genes and common polymorphisms in IL-4 may also affect expression of the gene. One of these common polymorphisms is a variable number of tandem repeat (VNTR) of 70 base pair which is situated in third intronic region of IL-4 gene. Three-repeat (3R) allele is the most frequent one while two-repeat (2R) allele is relatively rare and 2R allele was reported to result in high IL-4 production (12–13).

Considering the fact that approximately 20 % of human neoplasms could be associated with infectious agents and the need for further studies evaluating the role of Human Papillomavirus (HPV) in colorectal tumors, the aim of the study also covered HPV prevalence in CRC and its potential effect in metastasis. HPV's oncogenic potential stems from targeting vital tumor suppressor proteins partly responsible for significant cellular processes such as cell cycle progression, DNA repair, apoptosis and cellular differentiation. HPV has two early viral oncogenes called as E6 and E7 which induce the degradation of protein p53 (P53) and retinoblastoma protein (pRb), respectively. Moreover, inactivation of pRb also interrupts the cell cycle and results in increased expression of tumor suppressor protein ($p16^{INK4a}$) (14–15). HPV consists of more than 100 types and certain variants are categorized as high-risk and low-risk types. High-risk types (16, 18, 31 and 45) have the potential to immortalize and transform cells while low-risk types (6, 11, 40, 42, etc.) generally reflect a benign profile (1). Despite the potential significance of HPV in tumorigenesis as mentioned above, contradictory results obtained with the association of HPV in colorectal tumors certainly necessitate further studies evaluating its risk effects in malignant behavior.

In this study, we aimed to evaluate the potential roles of KRAS/NRAS proto-oncogenes, IL-4 VNTR variants and HPV prevalence (including high-risk oncogenic types (HPV16 and HPV18) in sixty colorectal tumors and explore their correlations with metastasis and other clinicopathological features. For this purpose, the patients were divided into categories as metastasis-present and metastasis-absent for a risk assessment.

Tab. 1. Clinicopathological features of the patients.

Characteristic		n	%
Gender	Female	26	43.3
	Male	34	56.7
Localisation	Not defined	3	5.0
	Rectum	15	25.0
	Rectosigmoid	6	10.0
	Sigmoid	14	23.3
	Descending Colon	1	1.6
	Left Colon	5	8.3
	Transverse Colon	2	3.3
	Ascending Colon	3	5.0
	Caecum	2	3.3
	Right_Colon	9	15.0
Region	Not defined	3	5.0
	Rectum	15	25.0
	Left Colon	26	43.3
	Transverse Colon	2	3.3
	Right Colon	14	23.3
Surgery	Biopsy	24	40.0
	Resection	4	6.7
	Left Hemicolectomy	11	18.3
	Right Colectomy	11	18.3
	Low Anterior Resection	6	10.0
	Extended Low Anterior Resection	3	5.0
	Laparoscopic Low Anterior Resection	1	1.7
Pathology	Adeno Ca	59	98.3
	Signet Cell Adeno Ca	1	1.7
Stage	pT1 N0 Mx L0 V0 R0	1	3.0
	yT2 N0 Mx. L0 V0 R0	3	9.1
	pT3 N1c Mx L0 V0 R0	20	60.6
	pmT4a N1b Mx L1 V1 Rx	5	15.2
	pT4b N0 Mx L0 V0 R0	4	12.1
Differentiation	Poorly Differentiated	9	17.0
	Moderately Differentiated	37	69.8
	Well-Differentiated	7	13.2
Metastasis	Positive	39	65.0
	Negative	21	35.0

Tab. 2. Association of metastasis with age.

	Metastasis						p
	n	positive		n	negative		
		Mean	SD		Mean	SD	
Age	39	64.67	11.42	21	73.76	12.63	**0.006**[a]

[a] – Student-t test

Tab. 3. Analysis of clinicopathological variables and investigated genes according to presence of metastasis.

			Metastasis		p
			positive	negative	
Gender	female	n	16	10	0.785[a]
		%	61.5	38.5	
	male	n	23	11	
		%	67.6	32.4	
Localisation	not defined	n	2	1	0.675[b]
		%	66.7	33.3	
	rectum	n	11	4	
		%	73.3	26.7	
	rectosigmoid	n	4	2	
		%	66.7	33.3	
	sigmoid	n	9	5	
		%	64.3	35.7	
	descending colon	n	1	0	
		%	100.0	0.0	
	left colon	n	2	3	
		%	40.0	60.0	
	transverse colon	n	1	1	
		%	50.0	50.0	
	ascending colon	n	3	0	
		%	100.0	0.0	
	caecum	n	1	1	
		%	50.0	50.0	
	right colon	n	4	5	
		%	44.4	55.6	
Region	not defined	n	2	1	0.644[b]
		%	66.7	33.3	
	rectum	n	12	3	
		%	80.0	20.0	
	left colon	n	16	10	
		%	61.5	38.5	
	transverse colon	n	1	1	
		%	50.0	50.0	
	right colon	n	8	6	
		%	57.1	42.9	
Surgery	biopsy	n	21	3	**0.002[b]**
		%	87.5%	12.5%	
	resection	n	1	3	
		%	25.0%	75.0%	
	left hemicolectomy	n	9	2	
		%	81.8%	18.2%	
	right colectomy	n	4	7	
		%	36.4%	63.6%	
	low anterior resection	n	3	3	
		%	50.0%	50.0%	
	extended low anterior resection	n	1	2	
		%	33.3%	66.7%	
	laparoscopic low anterior resection	n	0	1	
		%	0.0%	100.0%	
Pathology	adeno ca	n	38	21	1.000[b]
		%	64.4	35.6	
	signet cell adeno ca	n	1	0	
		%	100.0	0.0	
Stage	pT1 N0 Mx L0 V0 R0	n	0	1	0.521[b]
		%	0.0	100.0	
	pT2 N0 Mx. L0 V0 R0	n	1	2	
		%	33.3	66.7	
	pT3 N1c Mx L0 V0 R0	n	8	12	
		%	40.0	60.0	

Materials and methods

Study population

Tumor specimens were obtained by a retrospective compilation from the records of the pathology department of the Goztepe Prof. Dr. Suleyman Yalcin City Hospital (Istanbul, Turkey) from 2021 and 2022. Patients with poor DNA quality of the tumor sample were excluded. Demographical/clinicopathological features (age, gender, tumor localisation, histological type, TNM (tumor node metastasis) stage, differentiation and metastasis data were collected for each patient from surgical and pathological records.

All parts of the study were conducted according to the Good Clinical Practices and Declaration of Helsinki. The study was approved by the Medical Ethics Committee of Goztepe Prof. Dr. Suleyman Yalcin City Hospital (No: 2022/0097).

DNA extraction

DNA was extracted from paraffin-embedded tissue samples using AmoyDx FFPE DNA Extraction kit (Amoy Diagnostics Co., Ltd., China) according to the manufacturer instructions. Approximately 5 µm tissue sections were cut from the paraffin block and were primarily subjected to deparaffinization. The liquid content was obtained after a minimum of 1 hour incubation at 56 °C depending on the size of the tissue and an additional 1 hour incubation at 90 °C and transferred into the column provided by the kit. Pure DNA was obtained by removing undesired non-DNA cell particles from the column with washing steps. Eluted DNA samples were stored at –20 °C for further molecular analyses.

KRAS, NRAS, EGFR, BRAF mutation analysis

DNA concentrations and purities were measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA) and dilution series of 2.5 ng/µl were carried out for further real-time polymerase chain reaction (real-time PCR) experiment series conducted with real-time PCR device (Bio-Rad CFX-96, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The analysis of KRAS mutations was performed using AmoyDx KRAS Mutation Detection Kit for real-time PCR. The AmoyDx KRAS Mu-

Tab. 3.

			Metastasis		p
			positive	negative	
Stage	pmT4a N1b Mx L1 V1 Rx	n	4	1	0.521[b]
		%	80.0	20.0	
	pT4b N0 Mx L0 V0 R0	n	2	2	
		%	50.0	50.0	
Differentiation	Poorly Differentiated	n	5	4	0.750[b]
		%	55.6	44.4	
	Moderately Differentiated	n	24	13	
		%	64.9	35.1	
	Well-Differentiated	n	4	3	
		%	57.1	42.9	
IL4VNTR	2R/2R	n	3	1	0.913[b]
		%	75.0	25.0	
	2R/3R	n	14	9	
		%	60.9	39.1	
	3R/3R	n	22	11	
		%	66.7	33.3	
IL4VNTR	2R/2R-2R/3R	n	17	10	0.488[a]
		%	63.0	37.0	
	3R/3R	n	22	11	
		%	66.7	33.3	
Alleles	2R	n	20	11	0.948[a]
		%	64.5	35.5	
	3R	n	58	31	
		%	65.2	34.8	
HPV	Negative	n	23	15	0.408[a]
		%	60.5	39.5	
	Positive	n	16	6	
		%	72.7	27.3	
KRAS	Negative	n	10	11	**0.040[a]**
		%	47.6	52.4	
	Positive	n	29	10	
		%	74.4	25.6	
NRAS	Negative	n	22	14	0.076[b]
		%	61.1	38.9	
	Positive	n	7	0	
		%	100.0	0.0	

[a] – Pearson's chi-square test, [b] – Fisher's exact test

tation Detection Kit is intended to assess KRAS mutation status in colorectal cancer patients and the assay can detect 19 somatic mutations in codons 12, 13, 59, 61, 117 and 146 of KRAS gene in human genomic DNA. AmoyDx NRAS Mutation Detection Kit was used for the detection of 16 somatic mutations in NRAS codons 12, 13, 59, 61, 117 and 146.

Upon demand by Oncology Clinics, EGFR and BRAF mutation status were evaluated in some samples with AmoyDx EGFR 29 Mutations Detection Kit (29 mutations in exons 18-21) and AmoyDx BRAF Mutation Detection Kit (V2) (Qualitative detection of V600E, V600E2, V600K, V600D, V600D2, V600A, V600R mutations in BRAF oncogene).

Genotyping of IL-4 VNTR

IL-4 VNTR polymorphism was analyzed with PCR using previously described primer pair (13). PCR was carried out in a volume of 25 µl containing 2 µl genomic DNA, 1x reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each primer pair and 2 units of Taq DNA polymerase (Thermo Fisher Scientific, MA, USA). The PCR protocol was: pre-denaturation step at 95 °C for 7 minutes, followed by 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 65 °C for 45 seconds, extension at 72 °C for 45 seconds, and a final extension step at 72 °C for 10 minutes. PCR products were loaded on 2.5 % molecular biology grade agarose gel stained with ethidium bromide, electrophoresed and analyzed with gel documentation system (SYNGENE Ingenius 3, England) to enable for the analysis of three-repeats (3R) allele: 253 bp and two-repeats (2R) allele: 183 bp.

HPV detection and genotyping

The samples were first evaluated for HPV presence with the GP5+/GP6+ consensus primer pair which amplifies a 150 bp fragment of the L1 HPV genomic region. In order to detect oncogenic HPV-16 and HPV-18 types, type-specific primers were used (16). PCR with the consensus primer pair involved 40 cycles: denaturation for 1 min at 94 °C, annealing for 1 min at 45 °C and extension for 1 min at 72 °C. A pre-denaturation step of 6 min at 94 °C was included and a final extension step for 10 min at 72 °C completed the reaction. PCR conditions for HPV-16 and HPV-18 genotypes were different in terms of annealing temperatures (57 °C for the HPV-16 specific primers and at 65 °C for the HPV-18 specific primers) and resulted in 152 and 216 bp bands, respectively.

Statistical analysis

IBM SPSS 22 statistics software was used for all statistical analyses in this study. Descriptive statistics are given as n (%) and mean ± standard deviation for categorical and numerical variables, respectively. The relationship between metastasis status and clinical parameters was determined by Chi-square or Fisher's exact test. In addition, odds ratio and related confidence intervals were calculated for significant relationships. $p < 0.05$ value was used to determine the statistical significance for all tests conducted.

Results

Characteristics of study subjects

The study group consisted of sixty patients with colorectal carcinoma, including 34 (56.7 %) men and 26 (43.3 %) women. The median age of the patients was 69 ± 12,53 years (Min: 37 –

Tab. 4. The frequencies of the analysed gene regions.

Gene	Frequency	n	%
KRAS	negative	21	35.0
	positive	39	65.0
NRAS	negative	36	83.7
	positive	7	16.3
EGFR	negative	14	100.0
	–	–	–
BRAF	negative	20	83.3
	positive	4	16.7
IL-4 VNTR	2R/2R	4	6.7
	2R/3R	23	38.3
	3R/3R	33	55.0
HPV	negative	38	63.3
	positive	22	36.7

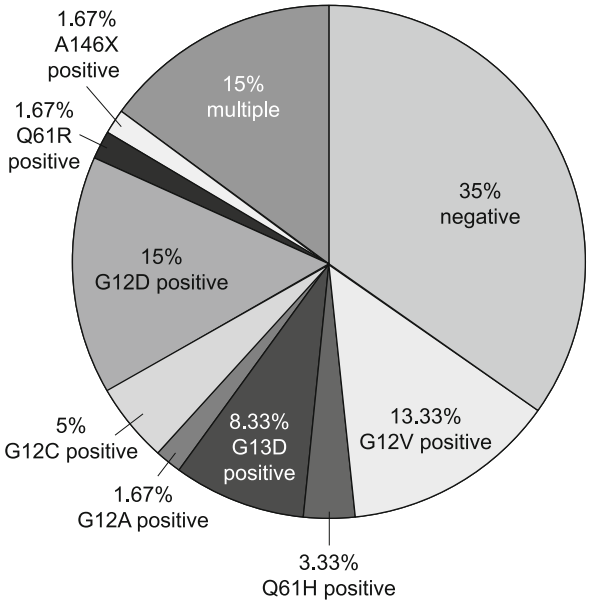

Fig. 1. The percentage of detected KRAS mutations in CRC patients. Multiple mutation slice (15%) shows cases carrying doublet/quartet mutations of different combinations. Doublet/quartet mutations: 1) G13D and G12S, 2) G12A and G13D, 3) G12V and Q61L, 4) K117X and A146X, 5) G12D and G12A, 6) Q61H and A146X, 7) G12D/G12R/G12V/G12C.

Max: 96). Thirty-nine cases (65 %) were positive for metastases. A very high percentage of the specimens were adenocarcinomas (98.3 %) while only one (1.7 %) specimen was signet ring cell carcinoma (SRCC). Moderately differentiated tumors were present with the highest percentage (69.8 %), this was followed by poorly differentiated tumors (17 %) and well-differentiated tumors (13.2 %). Detailed clinical characteristics of patients are summarized in Table 1. From the demographical parameters investigated, though gender was not associated with metastasis ($p > 0.05$), age showed association with metastasis ($p = 0.006$). The mean age of the patients with metastasis was lower than of the ones metastasis negative (Tab. 2). From the clinicopathological variables analyzed, biopsy specimens were associated with metastasis ($p = 0.002$) (Tab. 3).

Distribution of KRAS/NRAS mutations and possible association with metastasis

As a clinical routine in KRAS/NRAS mutation screening for patients with CRC, all of the patients were first screened for KRAS mutations and the negative ones for KRAS mutation were further screneed for NRAS mutations. Upon the demand by Oncology Clinics, EGFR and BRAF mutation screenings were performed in some limited cases but these were not included in the statistical analysis not to create a confounding effect. From 14 cases analyzed for EGFR mutations, all were negative. From 24 cases analyzed for BRAF mutations, 4 cases were BRAF mutated and the mutation type was V600E in all (Tab. 4). From 60 CRC patients screened for KRAS mutations, 39 people (65 %) were positive for KRAS mutations. The most frequent mutation type was G12D (15 %), followed by G12V (13.33 %). G13D mutation was ranked as the third most frequent mutation with 8.33 % (For single mutations). Besides, some patients were characterized with multiple mutations (15 %) (In 7 cases, doublet mutations were observed and one case carried quartet mutations). In one case, the coexistence of 2 of the examined KRAS mutations (G13D, G12S) was found. In 2 cases, the coexistence of 2 KRAS mutations (G12A, G13D) was found. In one case, the coexistence of G12V+Q61L mutations was observed. Another case carried doublet mutations of K117X and A146X. One case carried doublet mutations of a different combination (G12D, G12A). In one case, the coexistence of 2 KRAS mutations (Q61H, A146X) was found. In only one case, the coexistence of quartet mutations (G12D, G12R, G12V, G12C) was observed. The percentage of detected mutations in colorectal cancer (CRC) patients is depicted in Figure 1.

KRAS-negative patients (n = 21, 35 %) were further screened for NRAS mutations. Besides, nearly half of KRAS-positive cases (n = 22) were also screened for NRAS as control. NRAS mutation types observed in a total of 43 cases screened were: G12D, Q61H, G12S, Q61L, A146T, and G13D. The most frequent NRAS mutation type was Q61L (9.30 %), followed by the equal amounts of G12D and A146T mutations (4.65 %). From 21 people negative for KRAS, only one case (4.80 %) was positive for NRAS mutation (G13D) while the other cases were NRAS-negative (95.2 %). Six cases positive for KRAS mutations were concomitantly NRAS-positive (27.27 %). Concomitant KRAS/NRAS mutations are depicted in Table 5.

KRAS mutation positivity was found to be significantly associated with metastasis. Metastasis risk increased 3.19 times in KRAS-mutated patients compared with KRAS-negative ones (OR = 3.19 (CI) = 1.043–9.758) (Tab. 3, Fig. 2).

Tab. 5. Concomitant KRAS/NRAS mutations.

KRAS mutation type	NRAS mutation type
G13D + G12S	G12D
G12D + G12R + G12V + G12C	Q61H + G12D + G12S + Q61L
Q61R	Q61L
G13D	A146T
K117X + A146X	A146T + Q61L
Q61H + A146X	Q61L

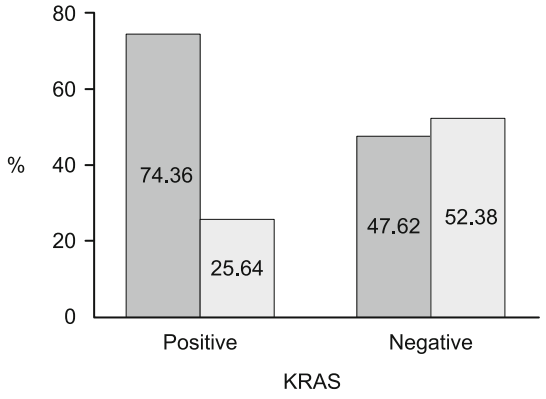

Fig. 2. Association of KRAS mutation with metastasis.

Analysis of IL-4 VNTR polymorphism and possible association with metastasis

The genotypic and allelic frequencies of the IL-4 intron 3 VNTR polymorphism were investigated in colorectal cancer patients. IL-4 VNTR genotypes can be classified as three-repeats (3R) allele: 253 bp and two-repeats (2R) allele: 183 bp. The distribution of the genotypes were: 3R/3R genotype was observed in 33 patients (55 %), 2R/3R genotype was observed in 23 patients (38.3 %) and the rare 2R/2R genotype was present only in 4 patients (6.7 %) (Tab. 4).

The possible association of IL-4 VNTR with metastasis was investigated like other factors analyzed. First, the genotypes were analyzed separately as 2R/2R, 2R/3R and 3R/3R, then also a combined version of the genotypes 2R/2R-2R/3R was investigated because of the rare 2R/2R genotype. IL-4 VNTR genotypes were not associated with metastasis separately or in combined genotype version ($p = 0.913$ and $p = 0.488$, respectively) (Tab. 3).

The allelic distributions of 2R and 3R were investigated in terms of constituting liability to metastasis. 2R allelic frequency was 64.5 % (n = 20) in metastasis-positive patients and 35.5 % (n = 11) in metastasis-negative ones, while 3R allelic frequency was 65.2 % (n = 58) in metastasis-positive patients and 34.8 % (n = 31) in metastasis-negative ones. Allelic distributions did not show association with metastasis ($p = 0.948$) (Tab. 3).

Besides metastasis association evaluation, the possible association of the investigated factors with each other was also investigated in detail. IL-4 VNTR was not found to be associated with metastasis ($p > 0.05$) (Tab. 3). There was no association of IL-4 VNTR genotypes with HPV and KRAS/NRAS positivity ($p > 0.05$).

HPV detection and possible association with metastasis

PCR with GP5+/GP6+ consensus primers was performed to evaluate the sequence of HPV. Type-specific primers were also used to detect oncogenic HPV-16 and HPV-18 types. Seven samples were positive for GP5+/GP6+ primer pair (11.66 %). The percentage of HPV-16 positive samples was 15 % (n = 9) while the percentage of HPV-18 positive samples was 11.66 % (n = 7). One HPV-18 positive sample was detected with GP5+/GP6+ primer pair, while the other HPV-16/HPV-18 positive samples were detected with type-specific primers. Our results show that other HPV types can also be present in the samples excluding HPV-16/HPV-18 but since we aimed to evaluate the highly oncogenic types, this was out of our scope. The percentage of HPV-16 positivity was a bit higher than HPV-18 positivity (15 % vs 11.66 %). In total, the frequency of HPVs in our colorectal cancer cohort was as high as 36.7 % (n = 22).

The association of HPVs with metastasis and IL-4 VNTR genotypes/KRAS/NRAS mutations was also evaluated. HPV positivity was not found to be associated with metastasis ($p > 0.05$) (Tab.3). When the possible associations of high-risk oncogenic types HPV-16 and HPV-18 with metastasis were evaluated, no statistically significant association was found ($p > 0.05$). Though HPV positivity was not associated with KRAS mutations, it was significantly associated with NRAS mutations; NRAS wild-type status acted as a protective factor by 7.5-fold for HPV negativity (OR = 7.5 CI = 1.080234–45.601) (Tab. 6).

Discussion

Since colorectal cancer (CRC) is one of the commonest reasons for deaths from cancer in both genders especially in developed countries, both the development of early diagnostic tools and molecularly-targeted therapies to control the disease and prevent possible metastases have great significance. Anti-epidermal growth factor receptor (EGFR) therapy necessitates RAS mutation status analysis and RAS wild-type (wt) patients can take ad-

Tab. 6. Association of HPV positivity with KRAS and NRAS mutations.

			HPV		p
			negative	positive	
KRAS	negative	n	16	5	0.166[a]
		%	76.2	23.8	
	positive	n	22	17	
		%	56.4	43.6	
NRAS	negative	n	27	9	0.028[b]
		%	75.0	25.0	
	positive	n	2	5	
		%	28.6	71.4	

[a] – Pearson's chi-square test, [b] – Fisher's exact test

vantage of the treatment. In most of the European countries, bevacizumab, cetuximab and panitumumab have been used as molecularly targeted drugs in advanced CRC. Bevacizumab is a monoclonal antibody against vascular endothelial growth factor (VEGF) and acts via inhibiting angiogenesis while the other two drugs are monoclonal antibodies against EGFR and act via inhibiting EGFR signaling pathway (5, 17). However, sometimes nearly half of patients who do not carry common KRAS codon 12 and 13 mutations do not response to anti-EGFR monoclonal antibodies and they need to be evaluated further. This evaluation may comprise other RAS mutations (rare KRAS mutations or NRAS mutations) and/or other downstream EGFR signaling factors such as BRAF (2, 5). Though BRAF mutations are rare, it may also be a good choice to consider it in analyses because of their potential roles in MAP-kinase (MAPK) pathway activation and contributions in key processes such as migration and apoptosis. Moreover, a distinct pattern of metastasis was reported in BRAF mutant tumors; they were characterized with higher rates of peritoneal and distant lymph node metastases and with less common rates of lung metastases (18–19). In our study, 60 CRC patients were screened for KRAS mutations and 39 individuals (65 %) were found positive for KRAS mutations. The most frequent mutation type in our cohort was G12D (15 %), followed by G12V (13.33%) and G13D (8.33 %). Multiple mutations in KRAS gene were also as high as 15 % (Fig. 1). In a total of 43 cases screened for NRAS mutations, different mutation types were observed as explained in the results section. The most frequent types were as follows: Q61L (9.30 %), G12D and A146T mutations (4.65 %). Most importantly, we observed a very clear association between KRAS mutation positivity and metastasis. This result is indirectly congruent with a study evaluating elderly metastatic colorectal cancer patients enrolled in TEGAFOX-E (cetuximab, oxaliplatin and uracil/ftorafur-UFT) phase II study. According to the results of this study, KRAS/NRAS wild-type status was independently associated with longer progression-free survival. Moreover, it was reported that patients could take maximum advantage of the treatment with cetuximab, oxaliplatin and UFT when KRAS/NRAS, BRAF and TP53 genes were wild-type (17). The frequency of KRAS mutations in Korean colorectal cancer patients were reported to be relatively low as 36.2 %, nearly half of the percentage determined in our population. The vast majority of KRAS mutations comprised of G12D, G12V, G13D and G13C, concertedly with our results. When KRAS codon 12 and 13 wild-type tumors were further screened for KRAS codon 61 and 146 or NRAS codon 12, the positivity rate was found to be 7.2 %. In discordance with our results, RAS mutation status did not reflect any association with clinical characteristics such as age, tumor location, metastasis (2). When driver mutations (KRAS, NRAS, BRAF, and PIK3CA) were evaluated in inoperable advanced Polish CRC patients, 45.1 % of the individuals had the examined mutations (KRAS codon 12, 13: 22.5 %; KRAS codon 61, 117, 146: 2.95 %; NRAS codon 12, 13, 61, 117: 3.94 %; BRAF: 6.87 % and PIK3CA: 3.93 %; doublet/triplet mutations: 4.91 %) (5). It is very obvious that the rate of KRAS mutations is less than found in our patient cohort. In line with our study, driver mutations were found more frequently in metastatic tissues. In Chinese colorectal cancer patients in whom prognostic values of KRAS, NRAS, BRAF and PIK3CA were investigated, KRAS mutations were found in 52.7 % of tumor samples (42.2 % of the mutations were in exon 2) and NRAS mutations were detected in 3.4 % of the samples. Moreover, KRAS exon 2 mutation was more frequent in older patients and associated with higher lymph node metastasis rate while KRAS exon 3 mutation displayed a less aggressive behavior and appeared mostly in lower TNM stage and smaller/less invasive tumors (20). In the analysis of Iranian colorectal cancer patients, KRAS mutations were detected in 28.7 % of the cases, mostly present in codon 12 exon 2 (72 %), then in codon 13 exon 2 (28 %) and most frequently observed mutation types were G12D and G13D. NRAS mutations in exon 2 (codon 12 or codon 13) were not detected. Also, KRAS mutations were reported to be more prevalent in tumors in colon than rectum (7). In Thai colon cancer patients, KRAS and NRAS mutation frequencies were reported as 47.2 % and 1.9 %, respectively and most of the KRAS mutations were in codon 12 (29.6 %). While KRAS G12D was the most common mutation, a higher frequency of KRAS codon 146 mutation (8.3%) when compared with other studies was also reported. In addition, compared with exon 2 and 3, KRAS exon 4 was associated with a better disease-free survival (21). In the analysis of KRAS/NRAS mutational profiles of metastatic colorectal cancer patients in Jordan, 48.42 % rate, mainly occurred in KRAS exon 2 mutations (the commonest type was G12D) was reported. NRAS exon 2 and 3 mutations constituted a ratio of 6.48 % while KRAS exon 3 and 4 mutations were present at a ratio of 10.87 % (8). In a very large Chinese colorectal cancer patient cohort with stages I-IV, frequencies of KRAS, NRAS and BRAF were reported as 46.4 %, 3.2 % and 3.5 %, respectively. While KRAS and BRAF mutations were independent risk factors for shorter overall survival in Stage IV tumors, the same risk vas valid for NRAS mutation in Stage I-II tumors (22). In another research also consisting of a very high number of Chinese colorectal cancer patients, evaluation of all driver mutations gave a sum of 48.9 % rate (KRAS: 36.1 %, PIK3CA: 10.2 %, NRAS: 3.9 %, BRAF: 2.9 %, HRAS: 0.9 % and EGFR: 0.9 %) (23). The frequency of KRAS mutations was reported as 41.5 %, with the majority in exon 2 (the most common type: G12D) in Danish colorectal cancer patients. The frequency of NRAS mutations was 4.2 % (mostly in exon 2, codons 12 and 13) and the common type identified was G12D in exon 2. BRAF mutation rate was 18.0 % (The most prevalent type: V600E substitution) and it was associated with high microsatellite instability which is predictive of mismatch repair deficiency (dMMR) (3). In a study conducted in locally advanced rectal cancer patients, mutation rates in KRAS, NRAS, BRAF, PIK3CA and TP53 were reported as 43 %, 9 %, 4 %, 9 % and 60 %, respectively. Compared with TP53/KRAS/NRAS wild-type tumors, patients who had a mutation in TP53 and either KRAS/NRAS were at risk for a worse 5-year progression-free survival (24). In a study evaluating KRAS/NRAS mutational profiles in Tunisian colorectal cancer patients, KRAS exon 2 mutation rates were reported as 41.7 % and the most prevalent types in codon 12 and 13 were G12D,

G12V, G12A, and G13D in coherence with the detected types in our study. NRAS mutation rate was 7.3 % and mostly occurred in exon 2. KRAS exon 2 mutations were found to be associated with increased age, left side colon and well differentiation. However, no association was reported between KRAS mutations and lymph node metastasis. NRAS mutations were characterized with early stages of cancer and absence of lymph node metastasis (4). This study reflecting a lack of relationship between KRAS mutations and metastasis is discordant with our findings. Besides, the authors (4) emphasized an important data which was also clarified in some previous reports; the mutually exclusive nature of KRAS (exon 2) and NRAS (exons 2, 3, and 4) mutations since none of the patients in their population harbored a simultaneous mutation. This situation was not valid in our analysis since concomitant mutations in KRAS/NRAS were detected as depicted in Table 5. Comprehensive data including analyzed mutation rates for KRAS/NRAS, the most prevalent mutation types in each, concomitant mutations, possible relationships of NRAS/KRAS mutations with metastasis and other clinicopathological factors were already presented in results section. As a very brief recapitulation and the most intriguing point of these data, in our colorectal cancer patient population, 65 % of the cases were detected as KRAS mutated. In contrary to infrequent NRAS mutations comprising nearly 2–4 % of tumors, the frequency of KRAS mutations can differ worldwide between 13 % and 66 % (7). Our study reflects that our population carries a very high proportion (65 %) of KRAS mutations which must be carefully evaluated in further studies and clinical practice.

As far as we know, our study is the first one evaluating the possible effect of IL-4 VNTR in metastatic process in colorectal cancer. In fact, the role of IL-4 on tumorigenesis seems like a contradictory issue which needs to be further evaluated. Though Chang et al (10) reported the inhibition of cell growth of colon carcinoma cell lines by IL-4 in a dose-dependent manner, an opposite report demonstrated the production of IL-4 by stem cell marker CD133+ cells to promote tumor cell survival (11). Some immunological biomarkers including Il-4 were investigated in colorectal cancer and it was reported that the possibility of the disease in later stage and metastasis process was very high when the serum levels of IL-4 $\geq$ 431 pg/ml and IL-7 $\geq$ 54 pg/ml (25). In a later immunohistochemical study conducted in colorectal cancer, expression profiles of Il-4, IL-13, IL-4R, and IL-13R proteins were investigated and they were all expressed in patients specimens. Moreover, patients with high expression of IL-4, IL-4R and IL-13R were characterized with lower lymph node metastases (26), a finding partially coherent with the report of Chang et al (10) since the authors pointed out to the dose-dependent nature of IL-4. In a more recent study evaluating the plasma levels of forty different inflammatory factors in CRC, high levels of CCL1, CCL20, CX3CL1 and IL-4 were reported to be associated with increased CRC-specific mortality (27). IL-4 seems to act as a double edge sword in tumorigenesis process since its role in tumor formation, metastasis and survival is heterogeneously complex and may also be dependent upon both dosage and tumor type. It is obvious that common polymorphisms in genes can affect gene expression and thus manage liability risk for the formation of different tumor types. One of these polymorphisms in IL-4 is a functional VNTR of 70 base pair in third intronic region of the gene and the relatively rare two-repeat (2R) allele was reported as a high producer of Il-4 (12). The possible effects of IL-4 intron 3 VNTR polymorphism were investigated in a few cancer studies in a case-control design. When compared with 3R/3R genotype, 3R/2R and 2R/2R genotypes of IL-4 VNTR (implying the possible risk effect of 2R allele) were found to be associated with approximately 1.9- and 3-fold increased gastric cancer risk, respectively in an Indian population (13). 2R/2R carrier genotypes (3R/2R and 2R/2R) were reported to be associated with late stage bladder cancer in northern Indian population (28) and 2R/2R genotype reflected a significant association for bladder cancer and tumor invasiveness in a Taiwanese population (29). Concertedly with previous studies, 3R/3R genotype of IL-4 VNTR was proven as a protective factor against breast cancer susceptibility in an Iranian population (30) while the effect of IL-4 VNTR variants was not observed in Jordanian breast cancer patients (31). In adult acute myeloid leukemia patients, the frequency of 3R alleles was higher than in controls and 3R/3R genotype was reported to be significantly associated with poor therapeutic response, higher susceptibility to disease recurrence and shorter overall survival and disease-free survival (32). This result was in contradiction with the study of Ahmed et al (33) who reported 2R/2R and 2R/3R genotypes as high risk factors for the development of leukemia. It is noteworthy to imply that all the above mentioned study designs were made in case-control series and made risk evaluation in terms of 2R and 3R alleles of IL-4 intron 3 VNTR. In our study design consisting of only patients, we therefore did not conduct a liability assessment for tumor formation but made a risk evaluation for metastasis. To the best of our knowledge, this is the first study analyzing the impact of IL-4 intron 3 VNTR in colorectal cancer metastasis. In our colorectal cancer study population, neither genotypes nor allelic distributions of IL-4 VNTR were found to be associated with metastasis. Considering the small population size, we strongly recommend further replication studies in larger populations to draw a definite conclusion.

As emphasized in the introduction part of the paper, colorectal carcinogenesis is a complex and multistep process involving both environmental/lifestyle factors and sequential changes at molecular level. Some other disputable issues in the development of colorectal cancer at microbiological level can be the change of microbiota and presence of viral DNA sequences, mainly focused on human papillomaviruses (HPVs). In this study, we also investigated the possible association of HPV presence with metastasis and other clinicopathological/molecular factors and prevalence of high-risk oncogenic types (HPV-16 and HPV-18) were also determined. HPV frequency in our colorectal cancer specimens was found as 36.7 %. The percentage of HPV-16 positivity was slightly higher than HPV-18 positivity (15 % vs 11.66 %). There was no association between the HPV presence and metastasis. However, a significant association previously not reported in literature was found between HPV and NRAS mutations. NRAS

wild-type status also acted as a protective factor (7.5-fold) for HPV negativity as mentioned in the results section and depicted in Table 6 (OR = 7.5 CI = 1.080234–45.601). As the potential etiological agent of colorectal tumorigenesis, several studies aimed to investigate the possible role of HPV viral involvement and/or high- risk oncogenic types HPV-16 and HPV-18 in pathogenesis process. The studies conducted up to now indicate the significance of HPV presence but also reflect a contradictory perspective from some points. The infection of normal colon mucosa with HPV-18 was found to be a risk factor in colorectal cancer development (34) though this result was not confirmed with a subsequent study which mainly emphasizes the significance of HPV-16; HPV-16 DNA was detected in 21.9 % of colorectal adenocarcinomas compared with matched nearby tissues (3.1 %) and no HPV-18 infection was detected (35). In the first study investigating the coexistence of KRAS mutations and HPV infection in colon cancer, although statistical analysis did not reveal an association, it was found that 56 % of HPV-positive tumors also harbored a KRAS mutation. Thus, it can be accepted that KRAS mutational activation is a common mechanism in colon tumorigenesis and HPV infection may also act as an additive step in malignant transformation (36). In the later study aiming to investigate the concurrence of HPV DNA and oncogene alterations, the researchers reported HPV frequency as 44 % and HPV-16 was the prevalent type though the ratio was quite close to HPV-18 as found in our study. C-myc amplication and KRAS mutations in cases were reported as 29.4 % and 30.7 %, respectively. No concurrence was present between KRAS/c-myc and viral DNA presence (1). In another study, a lower frequency (14.2 %) of HPV DNA with HPV-16 as the most prevalent type was reported and HPV positive patients were characterized by younger age (37). In the study of Sun et al (38) conducted with rectal tumor specimens, HPV infection was as high as 73.33 % and not detected in normal rectal mucosa. HPV infection was associated with age and lymphatic metastasis and also coexisted with mutations of APC (56.4 %) and KRAS (43.6 %). In contrast to what the above mentioned studies reported, none or negligibly small frequency (1 %) of HPV was reported in Iranian colorectal tissue specimens (14, 39). In Indian colorectal adenocarcinoma patients, HPV DNA was detected in 36.5 % of tissues, HPV-18 being the prevalent type and KRAS codon 12, 13 mutations were detected in 36.5 % of the cases and though statistically not significant, HPV positive cases harboured higher KRAS codon 12 (47.05 %) and 13 mutations (30.5 %) (40). In another study, a lower frequency (13 %) of HPV, HPV-16 being the most prevalent genotype was reported in colorectal carcinomas (15). In the study of Karbasi et al (6), HPV-16 and HPV-18 prevalences were reported as 10.5 % and 23.6 %, respectively and compared to adjacent normal tissue, lower expression of E6 gene in HPV-positive tumors was reported. Since both HPV and high-risk oncogenic types of HPV prevalences seem to fluctuate in different populations, this issue needs to be further evaluated in different and larger populations worldwide to reach a consensus for the role of HPV in colorectal tumorigenesis. Though our investigation did not reveal an association between metastasis and HPV DNA presence, HPV prevalence in our colorectal tumor samples was not at a negligible level (36.7 %). Moreover, our main aim was to analyze the possible relationship between the analyzed molecular/viral factors and metastasis and therefore the study design was not a case-control series. Further studies planned in a case-control series design may help to clarify the possible association between HPV presence and development of colorectal tumorigenesis in our population. Most importantly, we detected a novel and previously not reported correlation between NRAS wild-type status and HPV negativity which must be taken into consideration in future study designs.

Our study has a limitation stemming from the low cohort number collected from a single center (All the same, while the initial patient number was more than twice, low quality DNA specimens were all excluded and the study was conducted with only very high-quality DNAs). Besides this limitation, our study has quite powerful tools such as follow-up information, firstly presenting the metastatic risk evaluation of KRAS/NRAS, IL-4 VNTR and HPV analyses together, to the best of our knowledge. Extended studies in which these combined approaches will be evaluated both in our population and worldwide will contribute to the literature to better manage colorectal cancer tumorigenesis from a molecular biological perspective.

Conclusion

It is inevitably obvious that personalized cancer therapy enriched with molecular biology techniques can pave the way in treatment approaches in clinical practice. When considering the molecular cascade of colorectal cancer, these approaches may be more significant from practical point of view and it is one of the best prospering models in molecular biomarker development in oncology. We obtained significant association of KRAS mutations with metastasis. Considering the fact that screening a wide panel of RAS mutations is crucial in molecularly targeted therapies, the studies conducted in this field must obviously be enriched and compared in different extended populations to reach to stratifications in biomarker analyses. Thus, the problems related with the resistance mechanisms to anti-EGFR therapies can better be solved and new molecular approaches may be on the agenda. Though our study is the first one analyzing the impact of IL-4 intron 3 VNTR in colorectal cancer metastasis, genotypes/allelic distributions of IL-4 VNTR were found not to be associated with metastasis. This certainly does not rule out the possible role of IL-4 VNTR in colorectal tumorigenesis and further replication studies must be conducted. Though not showing association with metastasis, we found a relatively high frequency of HPV presence and most importantly there was a clear relationship between NRAS and HPV. As a result, our study offers a quite comprehensive evaluation of KRAS/NRAS, IL-4 and HPV analyses in colorectal cancer patients and metastasis risk analysis. The ongoing molecular/microbiological researches in this field could strengthen individualized patient-oriented therapy approaches in oncology.

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