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Cancer-Testis Antigen (MAGE-A3) Gene Expression in Colorectal Cancer Patients

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Abstract **Background:** Colorectal cancer is a leading cause of cancer-related mortality worldwide. Early detection is essential for effective treatment. Cancer-testis antigens, such as MAGE-A3, have been identified as potential biomarkers in cancer progression and therapy. This study aimed to evaluate the mRNA expression of MAGE-A3 in blood samples from patients with colorectal cancer (CRC) and non-malignant colorectal diseases (CRD). **Methods:** A total of 60 patients (30 CRC, 30 CRD) and 30 healthy controls were included. Ages (16-81), whose samples were collected from the Medical City Directory hospitals (GIT and Liver diseases Teaching hospital, Baghdad Teaching Hospital and Oncology Hospital) from 1st of November 2024 to 31 January 2025. and RNA was extracted using TRIzol™ reagent. Quantitative PCR (qPCR) was used to measure MAGE-A3 expression, normalised for the healthy control group. Statistical comparisons were made between CRC and CRD groups. The proportion of patients exhibiting gene expression levels above one is elevated in CRC. **Results:** sex distribution was not statistically significant ($p>0.05$), adenocarcinoma was the dominant type of cancer among such cases, a subgroup of patients (86.7%), The grade was moderately differentiated among the majority of patients (83.3%), MAGE-A3 mRNA expression was higher in CRC than CRD (52.6% vs. 47.4%; Likelihood Ratio = 4.317, df = 1, $p = 0.038$). **Conclusion:** MAGE-A3 mRNA expression is markedly elevated in CRC and may serve as a potential diagnostic biomarker for distinguishing malignant from benign colorectal conditions.

Key Words Colorectal Cancer, Colorectal Diseases, Cancer-Testis Antigens, MAGE-A3, Gene Expression

INTRODUCTION

Colorectal cancer (CRC) arises from a complex interaction of genetic, immunological, environmental and microbiome-related factors. It typically originates from adenomatous polyps formed by intestinal stem cells and may result from genetic alterations, such as mutations in the Adenomatous polyposis coli gene (associated with familial adenomatous polyposis) or mismatch repair genes, as observed in Lynch syndrome [1]. The progression of Colorectal cancer, referred to as “multi-step carcinogenesis”, involves a series of progressive alterations [2]. Globally, CRC is one of the most prevalent malignancies, differences in incidence and outcomes among populations are likely influenced by variations in lifestyle and environmental variables associated with CRC [3]. Also, epigenetic regulation serves as a vital molecular marker in cancer, playing a significant role in the pathophysiological interaction between genetics and the environment [4]. In Iraq, colorectal cancer is the second most prevalent malignancy [5].

Cancer Testis Antigens (CTAs) exhibit varied expression profiles in various types of malignancies such as melanoma, prostate, lung, breast, GIT, renal cell adenocarcinoma, immune cell malignancies and Colorectal. Melanoma-associated antigens (MAGE), belong to the cancer/testis antigen CTA family and were first discovered by Weon and Potts [6] comprise a minimum of 55 members, categorized into sub-families: MAGE-I comprises MAGE-A, B and C, whereas MAGE-II includes MAGE- D, E, F, G, H and L.

The MAGE-A gene is located at the chromosomal location Xq28 and it has been associated with certain hereditary disorders, such as dyskeratosis congenita [7] and

its expression is usually linked to poor prognosis and metastasis in cancer patients [8]. Melanoma-associated antigen A3, it is normally expressed in germline and trophoblast cells but demonstrates aberrant expression in some tumors, including melanoma, brain tumor, breast cancer, lung cancer and colorectal cancer due to hypomethylation of its promoter region [9]. The main player in MAGE-A3 hypomethylation is DNA methyltransferase enzymes (DNMTs), which mainly work in the gene promoter area. In colorectal cancer, demethylation of the MAGE-A3 promoter facilitates the advancement of cancer cells. The expression pattern of MAGEA3 in Colorectal cancer correlates with patient prognosis, particularly in microsatellite-stable (MSS) subtypes, its expression is associated with immune response and might be a possible immunotherapy target in MSS CRC patients [10].

Also, Histone modifications critically regulate MAGE-A3 expression in colorectal cancer (CRC) cells. Active transcription is linked to elevated histone H3 acetylation and H3K4 tri-methylation at the MAGE-A3 promoter, while restrictive modifications such as H3K27 tri-methylation are associated with gene silencing, indicating a role for histone alterations in the epigenetic control of MAGE-A3 [11].

The expression of CT antigens such as MAGEA3 in a subset of CRC patients induces readily detectable T cell responses, which could be boosted by active vaccination in a small subset of CRC patients [12].

Research shows that in colorectal cancer patient samples, MAGE-A3/6 expression is associated with reduced AMPK activity and enhanced mTOR signalling, underscoring the oncogenic function of MAGE-A3/6 in tumour metabolism [13].

Furthermore, MAGE-A3 specifically reduces the production and release of vascular endothelial growth factor in colorectal cancer via the mechanistic target of rapamycin pathway, without influencing other angiogenic factors. It also decreases mitochondrial function, facilitating tumor growth [8].

MAGE-A proteins in clinical tumor samples were observed to inhibit p53 activity, even with the presence of wild-type p53. Marcar *et al.* [14] established that MAGE-A antigens impede p53 functionality by blocking its association with chromatin, thus diminishing p53-mediated apoptosis and cell cycle arrest. These data underscore the therapeutic potential of addressing the p53/Mage-A interaction.

MAGE-A3 has emerged as a potential biomarker for predicting responses to immunotherapy. Szincsak *et al.* [15] performed a systematic evaluation and meta-analysis of machine learning models utilizing tumors RNA expression data from gastrointestinal cancer patients undergoing treatment with immune checkpoint inhibitors.

Despite research on MAGE-A3 in colorectal cancer in other populations, its expression has not yet been investigated in Iraqi patients. this is the first study to evaluate MAGE-A3 mRNA expression in blood samples from Iraqi CRC patients, providing novel molecular insight and assessing its potential as a diagnostic biomarker.

The objectives of this study to evaluate MAGE-A3 mRNA expression in blood samples from patients with colorectal cancer (CRC) and non-malignant colorectal diseases (CRD). And to assess the potential of MAGE-A3 as a diagnostic biomarker for distinguishing malignant from benign colorectal conditions.

Study Design and Blood Sample Collection

A case-control study was conducted from 1st of November 2024 to 31 January 2025 at the Department of Microbiology, College of Medicine, Al-Iraqia University.

Two groups were categorised from 60 patients suffering from GIT problems: The first one, the CRC group (n = 30), patients who are diagnosed with confirmed colorectal cancer and didn't start therapy, the second one, the CRD group (n=30), patients who suffer from other Colorectal diseases. Healthy control samples (n = 30) were used as the calibrator group to normalise gene expression data from CRC (n = 30) and CRD (n = 30) samples. All groups were with ages ranging between (16-81) years old, of both sexes. Blood samples were collected from participants selected from major hospitals in Baghdad, including the Medical City Directory hospitals (GIT and Liver Diseases Teaching Hospital, Baghdad Teaching Hospital and Oncology Hospital), representing the typical patient population for colorectal diseases.

Exclusion Criteria

Excluded from the study were patients who stopped participating because they underwent chemotherapy, passed away or decided not to continue and hemolysis samples.

Sample Collection and RNA Extraction

RNA Extraction: Total RNA was extracted using TRIzol™ Reagent (Invitrogen, USA; Cat. No. 15596026) following the manufacturer's instructions. Briefly, 600 µL of TRIzol™ was added to 0.5 mL of blood in a 1.5 mL microcentrifuge tube, followed by 0.15 mL chloroform for cell lysis. Samples were incubated at 25°C for 20 min and then centrifuged at 12,000×g for 15 min. The mixture separated into a lower red phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The aqueous phase containing RNA was carefully transferred to a new tube. RNA was precipitated by adding 0.45 mL isopropanol, incubated at 25°C for 20 min and centrifuged at 12,000×g for 10 min. The RNA pellet was washed with 0.75 mL of 75% ethanol, vortexed and centrifuged at 7,500×g for 5 min. The supernatant was discarded and the pellet was air-dried for 15 min, then resuspended in 50 µL RNase-free water and incubated at 60°C for 15 min using a thermomixer. RNA

quantity and purity were measured using a Nanodrop spectrophotometer and integrity was confirmed by agarose gel electrophoresis.

Quantitative Real-Time (RT-PCR)

Following the manufacturer's instructions, total RNA was converted into cDNA using the EasyScript® First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China; Cat. No. AE301-02). The cDNA synthesis process involved incubating the mixture at 25°C for 10 minutes to allow random primers to anneal, followed by 42°C for 15 minutes to activate reverse transcriptase and enable binding of Oligo(dT) primers and finally at 85°C for 5 seconds to terminate enzyme activity.The resulting of cDNA was stored at -20°C until use.

Real-time PCR was done using SYBR Green detection with a special kit on a Rotor-Gene Q Real-Time PCR System. The PCR reaction mixture (20 µL) contained SYBR Green Master Mix, forward and reverse primers, cDNA template and nuclease-free water. Each sample was duplicate for both the target and reference genes.

The thermal cycling conditions included enzyme activation at 95°C for 60 seconds (1 cycle), denaturation at 95ºC for 15 seconds, extension at 60ºC for 30 seconds for 45 cycles, followed by melt curve analysis from 60 to 95ºC.

The 2^(-ΔΔCt) method [16] was used for relative quantification, with GAPDH serving as the internal reference gene to normalise threshold cycle (Ct) values. ΔCT was calculated by subtracting the Ct of GAPDH from the Ct of MAGE-A3 and ΔΔCT was obtained by subtracting the ΔCT of the control samples from the ΔCT of the experimental samples. Relative expression levels were expressed as 2^(-ΔΔCT) to ensure accurate quantification of MAGE-A3 expression.

Specific primers for MAGE-A3 and the housekeeping gene GAPDH were designed and obtained from Macrogen® (Korea) (Table 1).

Ethical Approval

The study protocol was approved by the Ethics Committee of Al-Iraqia University (Approval ID: FM.SA/150 2025/4/27). Written informed consent was obtained from all participants prior to sample collection.

Statical Analysis

Data were entered, verified and analysed using computer software programs of Statistical Package of Social Science (SPSS) version 26 and STATISTICA version 9. Descriptive statistics including frequency, distribution tables, number and percentages for qualitative data as well as mean, standard deviation and range for quantitative data were employed, Comparisons of MAGE-A3 expression among the study groups (CRC, CRD and healthy controls) were performed using one-way ANOVA, assuming normal distribution and homogeneity of variance. Chi-square tests were applied to assess differences in categorical variables, such as sex distribution and expression positivity, while the Likelihood ratio test was used as an alternative when Chi-square assumptions were not fully met. A p-value of <0.05 was used for determining statistical significance throughout study.

RESULTS

Demographic Data: Table 2 summarizes the age and gender distribution of study participants. Overall, most participants were aged 30-60 years (≈69%). The CRC group was older on average than the CRD and control groups (56.5±14.6 vs. 38.6±13.3 and 47.7±11.6 years; $p<0.001$), with a notable proportion over 60 years. Gender distribution was balanced across groups, with males slightly predominating in the CRC and control groups, while females were more frequent in the CRD group. This difference was not statistically significant ($p = 0.392$), reflecting successful matching of study groups.

Among the colorectal disease (CRD) group, acute inflammatory disease (AID) was the most frequent condition, affecting approximately one-quarter of patients (23.3%). This was followed by ulcerative colitis (20%), chronic inflammation and congestion (each 13.3%) and polyps and adenoma (10%). Less common conditions included diverticula, lipoma and Crohn's disease (each 3.3%) (Figure 1).

The type of cancer among colorectal cancer subgroup of cases sample, adenocarcinoma was dominant type of cancer among such cases subgroup of patient (86.7%) (Figure 2).

The grade of tumors among cases subgroups of colorectal cancer were moderately differentiated among majority of patients (83.3%) followed by 13.3% were poorly differentiated and only 3.3% of tumors were well differentiated (Figure 3).

MAGEA3 Gene Expression

The proportion of patients with MAGE-A3 expression ≥1 was significantly higher in the CRC subgroup compared with the CRD subgroup (52.6% vs. 47.4%, respectively; likelihood ratio = 4.317, df = 1, $p = 0.038$) (Table 3, Figure 4).

The CRC subgroup exhibited a significantly higher proportion of patients with MAGE-A3 expression ≥1 compared with the CRD subgroup.

Table 1: Primers Used and Designed in this Study

Gene/Primer	Sequence (5′ → 3′)	Source
MAGE-A3 Forward (MAGE-A3_F)	5-GTTTCCACTGCCTCCTGTGAC-3	macrogen® (Korea)
MAGE-A3 Reverse (MAGE-A3_R)	5-GACGCTCATTCAACCATCCGT-3	macrogen® (Korea)
GAPDH Forward (GAPDH-F)	5-GTCTCCTCTGACTTCAA-3	macrogen® (Korea)
GAPDH Reverse (GAPDH-R)	5-ACCACCCTGTTGCTGTA-3	macrogen® (Korea)

Table 2: Age and Gender Distribution of CRC, CRD and Control Groups (n = 90)

Characteristics	Study groups				
	CRC (n = 30)	CRD (n = 30)	Control (n = 30)	Total (n = 90)	Significancy
Age (years)					
Mean±SD	56.50±14.562	38.57±13.268	47.73±11.585	47.60±14.980	F = 13.857, df: (2, 87)
Range (min-max)	55 (26- 81)	48 (16- 64)	44 (22- 66)	65 (16- 81)	p = 0.000[a]
Age (In groups)					
<30	2 (6.7)	8 (26.7)	3 (10)	13 (14.4)	Likehood Ratio: 12.272, df: 4,
30-60	18 (60)	20 (66.7)	24 (80)	62 (68.9)	p = 0.014[b]
>60	10 (33.3)	2 (6.7)	3 (10)	15 (16.7)	
Gender					
Female	13 (43.3)	17 (56.7)	12 (40)	42 (46.7)	x^2: 1.875, df: 2,
Male	17 (56.7)	13 (43.3)	18 (60)	48 (53.3)	p = 0.392[c]

Table 3: Distribution of Study Cases According to MAGE-A3 Gene Expression (n = 60)

Genetic Expression of MAGE-A3	Study's cases subgroups				Total
	CRC (30)		CRD (30)		
	N	%	n	%	
<1	-	-	3	100	3
≥1	30	52.6	27	47.4	57

Likehood Ratio: 4.317, df: 1, p = 0.038

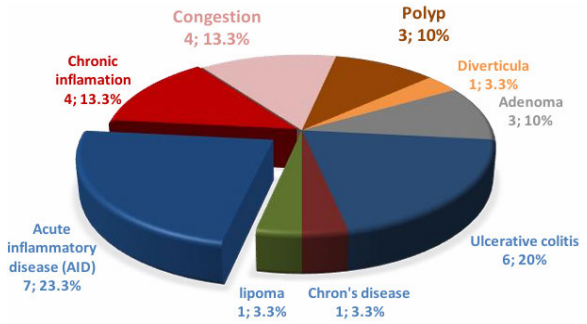

Figure 1: Distribution of Benign and Inflammatory Conditions in the CRD Subgroup (n = 30)

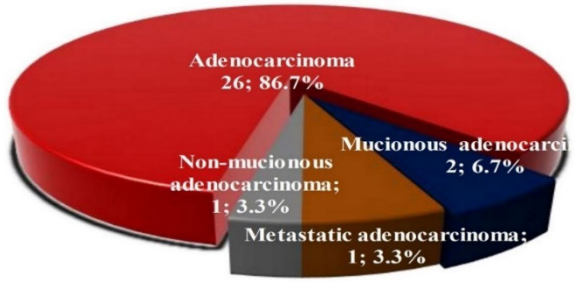

Figure 2: Distribution of Type of Cancer Among Cases Subgroup of Colorectal Patients (n = 30)

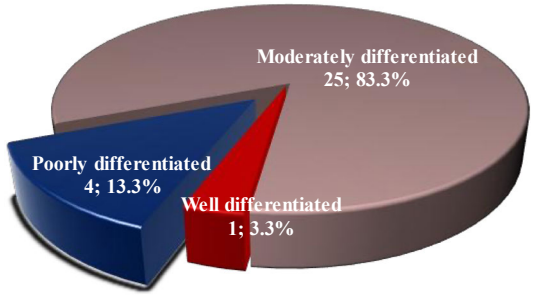

Figure 3: Distribution of Grade of Cancer Among Cases Subgroup of Colorectal Patients (n = 30)

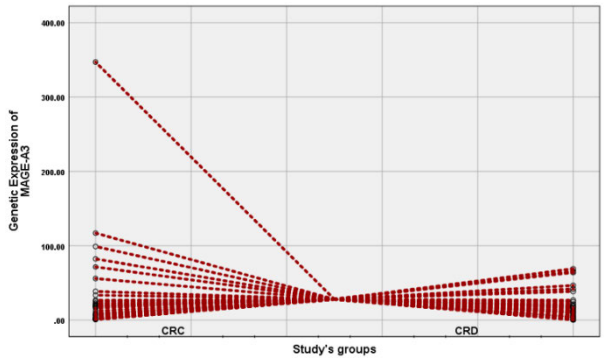

Figure 4: MAGE-A3 Gene Expression Among Study Subgroups (n = 60)

DISCUSSION

The results showed that MAGE-A3 expression is significantly elevated in peripheral blood of colorectal cancer (CRC) patients compared to those with non-malignant colorectal diseases (CRD), suggesting that MAGE-A3 mRNA is highly upregulated in colorectal cancer and plays a role in tumor development or progression.

The source of the MAGE-A3 mRNA in the CRC patients is likely from Circulating Tumor cells (CTCs) [10]. Or exosomes circulating in the peripheral blood [17].

The source of MAGE-A3 mRNA in CRD cases may be because those benign cases may suffer from expressing methylation abnormalities in MAGE-A3. This suggests that even in non-cancerous tissue, loss of normal DNA methylation patterns, a hallmark of early neoplastic change, can lead to inappropriate expression of genes like MAGE-A. These changes might not be sufficient for malignancy but they indicate molecular instability [18].

A study found that MAGE-A3 was detected not only in patients with lung cancer but also in a subset of patients with non-malignant lung diseases, including conditions such as tuberculosis and other chronic inflammatory diseases. These findings indicate that MAGE-A gene activation is not entirely specific to

malignant transformation but may also occur in response to chronic inflammation, regenerative hyperplasia or epigenetic dysregulation [19].

Another study found MAGE-A3 antigen in airway epithelial cells of chronic smokers without lung cancer, suggesting tobacco carcinogens alter gene methylation patterns [20].

A previous study on Prostate cancer showed high-grade prostatic intraepithelial neoplasia, Benign prostatic hyperplasia, indicates that some level of MAGE-A3 expression is present in Benign prostatic hyperplasia but at low or minimal levels and significantly less than in malignant tissue [7].

Benign prostatic hyperplasia samples found limited expression of the MAGE-A3 and MAGE-A4 in 3 out of 20 specimens, 15% of benign cases had MAGE-A3/4 immunoreactivity. In all positive samples, antigen expression was less than 10% and weak, suggesting MAGE-A3/4 antigens are predominantly associated with malignant transformation [21]. In previous study quantitative PCR was used to determine MAGE-A3 expression found in 28% of tumors, with a tumor vs. testis expression ratio above 0.1% [22]. Also, Immunohistochemical studies revealed that MAGE-A3 co-expresses with mismatch repair proteins MSH6 and PMS2, suggesting a link between MAGE-A3 expression and the tumour's microsatellite stability [23]. Promoter hypomethylation of MAGE-A3 was significantly associated with gene reactivation in colorectal cancer; 77% of colorectal cancer tissues showed hypomethylation of MAGE-A3, compared to only 6% in normal tissues, indicating that hypomethylation plays a key role in the gene's activation in cancer [21]. Another study investigated the correlation between MAGEA3 and resistance to bevacizumab in CRC, suggesting that (Melanoma-associated antigen A3) it could be a biomarker for bevacizumab resistance and patient prognosis in Colorectal cancer [8].

An early study of clinical colorectal cancer (CRC) specimens indicated that MAGE-A3 was present in approximately 20% of tumors, absent in adjacent normal tissues and more prevalent in cases with liver metastases, suggesting its potential role in tumour progression and as a target for immunotherapeutic strategies [24].

Co-expression of MAGE-A3 and MAGE-A4 antibodies in colon, lung and bladder cancers highlights their tumor-specific expression and possible use in co-immunotherapy with PD-L1 [25].

Another study identified a higher prevalence of MAGE-A3 and MAGE-A4 expression (13% and 15%, respectively) in human colorectal cancer compared to other CT genes [12].

MAGE-A3 expression detected via multimarker qRT-PCR in sentinel lymph nodes and blood samples revealed micrometastases not identified by routine histology, emphasizing its potential as a prognostic marker [26].

Additionally, activated B cells expressing MAGE-A3, facilitated by modified viral vectors, induced cytotoxic T cells capable of targeting CRC cells [27].

A study in Saudi Arabia identified MAGE-A3 mRNA expression in 60% of CC patients and hypomethylation in the promoter regions of MAGE-A1, MAGE-A3 and MAGE-A4 genes, suggesting their up-regulation may play a role in CC development and progression [28].

Other studies suggest that MAGE-A3, along with IL-1β and IDH1, may serve as candidate biomarkers for immunotherapy due to their elevated expression in colon cancer tissues [29].

CONCLUSIONS

This study highlights the significant upregulation of MAGE-A3 mRNA in patients with colorectal cancer (CRC) compared to CRD. Age was significantly associated with colorectal cancer and disease (P = 0.000), while sex differences were not significant. Among CRC cases, adenocarcinoma was the predominant type, with most tumors moderately differentiated. Gene expression above one was significantly higher in the CRC group than the CRD group, suggesting its potential as a diagnostic biomarker for CRC.

Future Recommendations

Future studies should include larger, multicenter cohorts, validate findings at the protein level, incorporate longitudinal follow-up and explore potential therapeutic implications.

Limitations

This study is limited by a small sample size, case-control design, single-country setting and lack of protein-level validation or long-term follow-up.

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