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ORIGINAL ARTICLE

Assessment of HER3 Gene Expression in Paraffin-Embedded tissues of Patients with Colon Cancer

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ABSTRACT
Colorectal cancer (CRC) is the second most prominent cause of cancer-related deaths in the world. Understanding the molecular pathway of CRC can provide some useful information for the development of therapeutic strategies. Hyperactivity of EGFR family has been reported in recent years, and HER3 has been proposed as a molecular marker of prognosis in many cancers. The purpose of this study was to investigate the expression of HER3 gene in patients with CRC and its association with advanced stages of disease. In this study, 20 samples of paraffin-embedded tissues taken from patients with age range of 24–84 years and 10 samples from normal subjects were analyzed. After sectioning and paraffin removal, RNA was extracted and cDNA synthesis was performed using the MMULV enzyme oligo(dT) and random hexamer primers. HER3-specific primers and GAPDH (as an internal control) were designed by using primer express software, and gene expression was measured by RQ method. Real-time PCR reaction analysis indicated an increase in the expression of HER3 gene in all samples compared with that in normal samples. HER3 expression levels in stages 3 and 4 were higher than those in patients in stages I and II, and it was also observed that this increased expression was directly related to aging. The results suggest that the expression level of HER3 gene increases in patients with CRC. Investigating the increased expression HER3 can be considered as an appropriate target for specific treatment, particularly in cases that are resistant to current treatments.
Keyword: *colorectal cancer, HER3, real-time PCR.*

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INTRODUCTION

Approximately 1.5 million people are diagnosed with colorectal cancer and more than 600,000 people lose their lives every year. Colorectal cancer is the third most common cancer in Iranian men and the fourth one in Iranian women [1]. Despite the advancements in the field of preventive medicine, it is also the second most common cancer in western countries [2]. Several different pathways, such as signaling pathways of WNT-B catenin, transforming growth factor (TGFβ), and epidermal growth factor receptor (EGFR) have been recognized in the context of colorectal cancer [3]. One of the important mutations involved in this disease is a mutation in the EGFR family. The family of human EGFRs comprises four types of tyrosine kinase transmembrane receptors, which are called HER1 (EGFR, ERbB1), HER2 (Neu, ErbB2), HER3 (ErbB3), and HER4 (ErbB4) [4]. Most of the tyrosine kinase receptors are formed by extracellular, transmembrane, and cytoplasmic tyrosine kinase domains. The activated tyrosine kinase receptor regulates most of the key processes, such as cell growth and survival. Impairment of tyrosine kinase receptor was observed in a variety of cancers. The gene encoding HER3 is located on 12q13 chromosome and encodes a protein with a molecular weight of 180 kD. [5]. Overexpression of HER2 can be seen in several malignancies, such as breast cancer, colorectal cancer, sarcoma cell carcinoma of head, and cancers of stomach, ovary, prostate, and bladder. Although HER2 does not have tyrosine kinase function and is unable to build homodimer, it can build heterodimeric structure with other HERs and get activated by the PI3K/KT pathway. Studies show that the expression of HER3 has a significant role in

carcinogenesis and is a logical target for anticancer therapy against the C-terminal of HER3 receptor. Considering the importance of early detection of colon cancer, it seems necessary to detect cancers in early stages and it would be an advantage in improving the treatment and prevention of the disease. Because of the high incidence of colon cancer and the importance of its early detection, evaluation of HER3 expression (as an early diagnosis marker) can be an effective step in controlling, prediction, therapeutic approach, and treatment. Until now, no screening test has been done by evaluating the HER3 level in the detection of cancer.

MATERIALS AND METHODS

In this study, 30 paraffin tissues (20 patients, 10 normal subjects) were selected for sectioning by a pathologist. Age range of the subjects was from 24 to 84 years, and the samples were collected from 2010 to 2012.

RNA extraction: After sectioning and preparation of the samples, RNA samples were extracted according to the protocol that was optimized in a previous study [6]. After extraction, the RNA quality was assessed by spectrophotometer and light absorbance, which was measured in 260/280 nm.

cDNA synthesis: Initially, 10 µl of RNA, 0.5 mMdNTP (CinnaGen, Iran), 0.2 µg of random hexamer (CinnaGen, Iran) , 0.5µg oligodT (CinnaGen, Iran) and MMULV 100U(CinnaGen, Iran) were mixed together, and the mixture was used at a final volume of 20 µl. The mixture was incubated for 1 hour at 42°C.

Specific primers for real-Time PCR:

GAPDH gene was used as an internal control. After preparing the sequences of GAPDH and HER3 on NCBI, the gene-specific primers were designed by primer express software. In order to verify the accuracy and specialization of primers, their sequences were blasted. Sequencing of primers is listed in table 1.

Table 1: characteristics of primers:

Amplicon Size	Sequences	Name
106bp	AGTGAGGCCAAGACTCCAAT	HER3-F
	ACTCCCAAACTGTCACACCA	HER3- R
85 bp	CCCACACACATGCACTTACC	GAPDH F
	TGCCTGTCCTTCCTAGCTCT	GAPDH R

Optimization of real-time PCR essential factors for GAPDH and HER3:

For this purpose, separate reactions were prepared for internal control gene and designed primers at a final volume of 20 µl. The reactions were performed in parallel on ABI 7500 instrument. Each reaction contained SYBR TM premix (1×), 0.4 mM of forward and reverse primers, and 2 µg of cDNA template. The real-time PCR reaction included 40 complete cycles at 95°C for 15 seconds and at 60°C for 1 minute. Dissociation curve analysis was used in order to verify the amplified fragments and the absence of non-specific amplification, primer-dimer formation, and pollution. After optimization of the test, the RNA molecules were extracted from all the samples and after obtaining the quality approval, cDNA synthesis was performed using the samples. After the reaction, raw data were obtained from the device as a CT, and the measurement of gene expression was performed by using ρρCt. Gene expression profile was plotted using Graphpad software.

RESULTS

Light absorbance of the extracted RNA was analyzed by spectrophotometer. After amplification of cDNA, all the tissue samples collected from the 20 patients and 10 normal subjects were analyzed by real-time PCR. Normal samples were used as reference samples for comparing the changes between the groups. In order to evaluate the specificity of the primers, a fluorescent dye (SYBR green) was used. The melting curves of HER3 gene (Figure 1) and GAPDH (Figure 1) were plotted individually by real-time PCR in order to ensure the amplification of specific primers and the absence of non-specific parts.

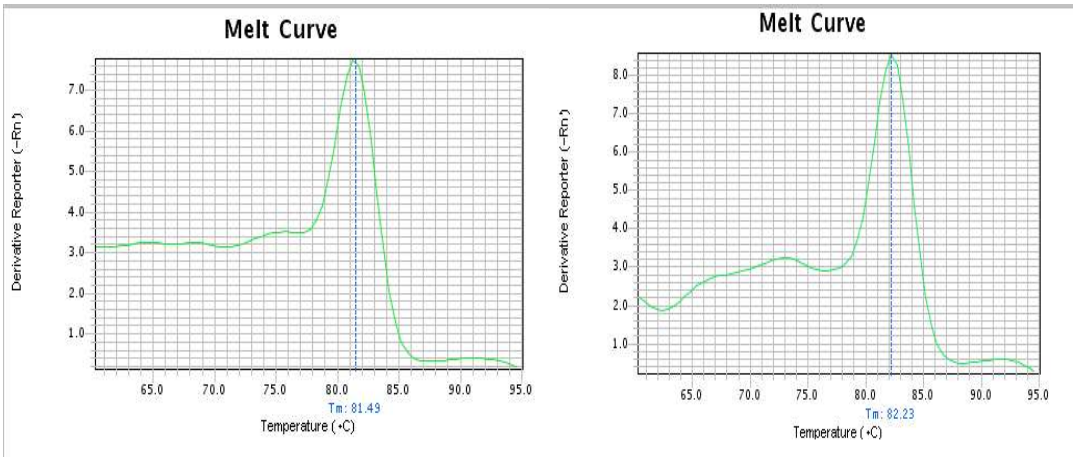

Figure1: Melting curve analysis of GAPDH and HER3 genes

After amplification, the CT of the samples was calculated by the device and it was converted to RQ (relative quantification) or expression level, and then ρρCt method was used for measuring the gene expression level. The expression levels of patient samples were expressed in comparison with those of the normal samples. The RQ of the samples was calculated by the device and the chart was drawn. The results were plotted by using Graphpad software (Figure 2).

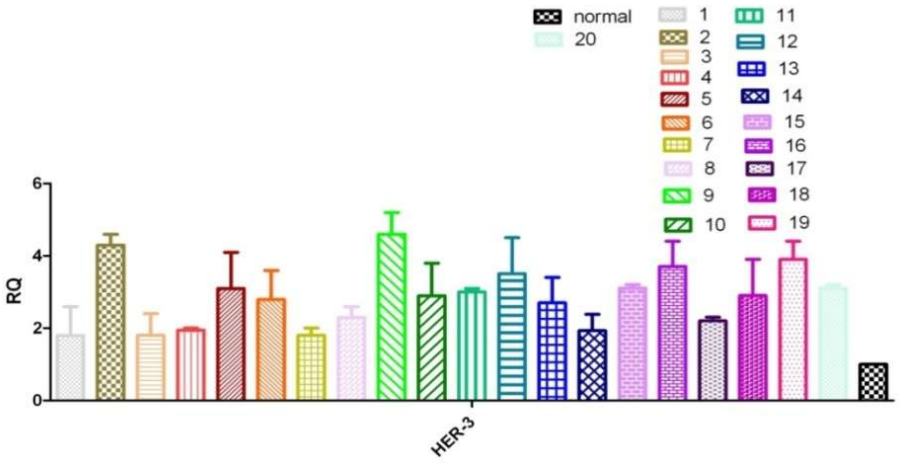

Figure 2: HER3 gene expression analysis in patients compared with control

As shown in Figure 3, the highest gene expression levels were observed for the sample numbers 2, 9, 16, and 19, all of which were in the third stage. Sample numbers 5, 10, 11, 12, and 13 were in stage 2 and they showed lower expression levels compared with those of the samples in stage 3. Sample numbers 1, 3, 4, 7, and 8 were in the first stage, whose expression levels dropped in comparison to those in stages 2 and 3. In this study, 10 normal samples were also used. For better evaluation, samples were normally recorded and their RQ was calculated by using mean values. Finally, the patient samples and normal samples were compared with each other. Evaluation of the results showed that HER3 gene expression was increased by 2.7 times in patients aged less than 50 years compared with that in the normal samples, and it was increased by 3.1 times in patients aged more than 50 years. Disease classification was done based on only the age group, and it was regardless of the stage of disease. Thus, we can infer that HER3 gene expression is directly related to aging.

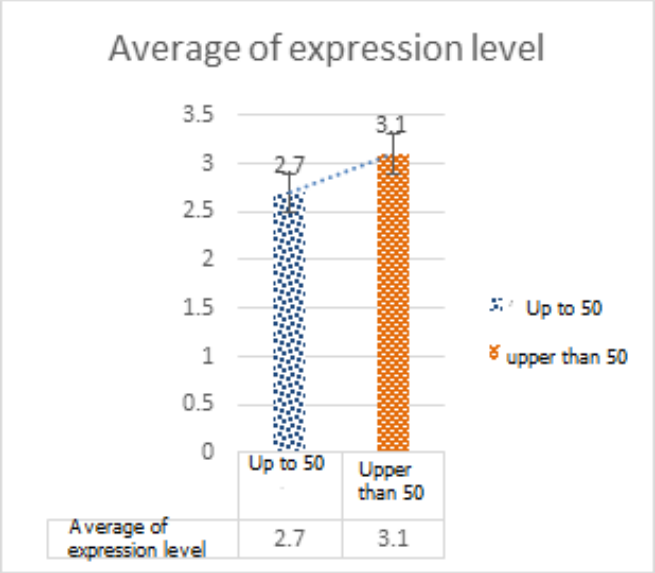

Figure 3: the average amount of HER3 gene expression of patients with 50 years old and above.

Regarding the stages of disease, it was observed that the average increase in HER3 gene expression was 1.2 ($p < 0.005$) in stage 1, 1.3 ($p < 0.005$) in stage 2, and 9.3 ($p < 0.005$) in stage 3 (Figure 4), which indicates that the expression of HER3 gene increases with the stage of the disease and the expression of this gene is associated with the disease progression. So it can be concluded that HER3 gene can be affected during the disease process and it can be an important candidate factor in the evaluation of cancer.

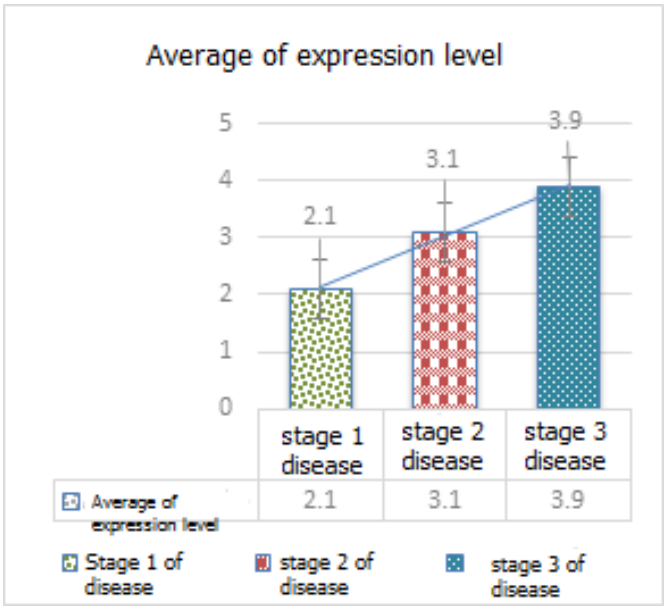

Figure 4: The average amount of HER3 expression in samples with regard to the stages of the disease

DISCUSSION

Colorectal cancer is one of the most common cancers worldwide. About 40–50% of newly diagnosed patients are affected with metastatic disease, and the average survival of patients is between 18 and 21 months [7]. Following the activation of HER3 gene, these proteins effectively accompanied with PI3K/AKT pathway which the functions of this pathway results in various cellular events including survival, motility and apoptosis. HER3 of the ErbB family has a weak or no tyrosine kinase activity. Because of these features, phosphorylation of HER3 occurs after Heregulin activation and heterodimerization by other members of the ErbB family. HER2 and HER3 interact with each other in human breast cancer, which results in the creation of carcinogenic dimer and initiation of tumor development. In fact, HER2/HER3 complex creates a very strong mitogen complex among the members of

the EGFR protein family. The dimer is quite effective in cell transformation and contributes to malignant properties of cancer cells. Kountourakis *et al*. [8] assessed the cytoplasmic and membranous expression patterns of HER3, respectively, in 18 samples (17%) and 30 samples (3.28%) by immunohistochemistry (IHC) method. HER3 cytotoxic expression was positive in all samples with an average grade of tumor ($p = 0.032$) and a higher age average ($p = 0.010$). These results are consistent with the present study. The expression of HER3 was increased in all the samples. Considering that they used IHC technique, the manner used in this study, had higher sensitivity. Scartozzi *et al*. [9] used IHC method for analyzing 84 cases of colorectal cancer and after statistical analysis, they proposed that HER3 is a predictive factor for the clinical outcome in K-Ras wild-type colorectal cancer treated with cetuximab. Combined analysis of HER3 and KRAS may be an effective strategy for a better selection of patients with colorectal cancer [9]. Khelwatty *et al*. [10] used IHC for studying the expression of HER family members in tumor samples from 86 patients with Dukes' and Dukes' (metastasis) colorectal cancers. Generally, 43%, 77%, 52%, and 92% of the cases were positive for HER3, HER2, EGFR, and HER4, respectively. In this study, 35%, 24%, 43%, and 18% of the cases were positive for EGFR, HER2, HER3, and HER4, respectively, and the members of HER family were expressed simultaneously. They declared that the simultaneous expression of HER family members in colorectal cancer confirmed the need for future evaluation of the predictive value for response to treatment with anti-EGFR mAbs [10]. As it was mentioned screening test like IHC (Immunohistochemistry) in comparison with real-time PCR which is the confirmatory test; does not have sufficient sensitivity and accuracy, so the advantage of this study was to use a more accurate, sensitive test instead of using such screening tests.

As noted above, the techniques used in most of the studies were based on protein identification techniques. In addition, the serology methods (because of resulting in false-positive and -negative results) are not very reliable, Therefore, because of the fact that the isolation and amplification of nucleic acids is more accurate and sensitive than antigen–antibody reactions, molecular techniques are considered a better alternative to these methods [11]. Furthermore, because of the unavailability of IHC kits and the non-conventional usage of IHC for HER3 gene expression analysis in Iran, it was not possible to assess the results by this technique. However, the overall results of real-time PCR were consistent with IHC results and those of real-time PCR studies conducted outside Iran. It was for the first time that the expression of HER3 gene was studied in patients with colorectal cancer in Iran and it is believed that more studies will be conducted on larger population with cancer in future. The 100% over expression of HER3 gene suggests the role of this gene in the development and progression of the disease compared with other members of this gene family. Further studies, especially cytological studies in evaluating HER3 dimerization, can confirm its role in the disease process. It appears that increasing age and stage of the disease are associated with the increased expression of HER3. Studies show that the prediction of outcome in patients with colorectal cancer is considered as a complex clinical problem. In addition, despite the massive improvement in cancer cure, a large limitation can be seen in the absence of definite treatment of patients, in which case, the use of multiple combined treatments is considered as an improving key. Disease progression is also associated with an increased risk of death. So the identification and use of new markers for diagnosis and utilizing them in treatment are necessary and are inevitable aspects, and it can be concluded that studies in this field have a special value. After analyzing the results of HER3 gene expression by Real Time PCR in all patient samples, the increased expression of HER3 was observed in comparison to 20 normal samples and the review of this gene in patients with colorectal cancer showed more expression in over 50 years old patients in comparison with under 50 years old. Thus, the HER3 gene is involved in various stages of colorectal cancer and it is increasingly expressed with the progression and stages of the disease.

CONCLUSION

The present study results show that HER3 gene expression is one of the prognosis molecular markers in colorectal cancer and by evaluation of its expression level, different strategies can be applied in patient treatment. In addition, by screening this gene, the right decision with regard to the kind of drug and treatment can be made along with the prediction of the tumor behavior, ultimately leading to appropriate treatment strategy. However, this method is not yet commonly used, and no valid diagnostic and therapeutic instructions have been proposed till now. Therefore, in line with the excellent features of this method, it seems necessary to conduct more studies for confirmation of its greater efficiency and accuracy.

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