Assessment of HER2 Gene Expression in Paraffin-Embedded tissues of Patients with colorectal cancer

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ABSTRACT

Introduction and purpose: Colorectal cancer is the fourth most common cancer in the world. It is the third largest risk factor for cancer individuals. The number of new cases is rising since 1975. Hyper activity of EGFR family have been reported in various cancers. The purpose of this study was to investigate the expression levels of HER2 in colorectal cancer and its possible association with advanced stages of disease.

Materials and methods: In this study 30 samples of colorectal cancer paraffin block and 10 non-tumor individual samples were collected. After de-paraffin, RNA molecules were extracted from samples by RNX plus solution, after that CDNA was synthetized by reverse transcription method and finally the relative HER2 gene expression was evaluated by Real Time PCR.

Result: In 30 cases of colorectal cancer compared with non-tumor samples, over expression of HER2 gene was observed. The increased expression compared with non-tumor samples observed in all patient samples and the amount of over expressed HER2 in patients with stage III was more than stage II and I expression levels were also increased by increasing the age. The observation showed that the increased expression of HER2 occurs from the early stages of the disease.

Conclusion: Due to the increased HER2 expression in colorectal cancer, it is clear that the HER2 assessment can be considered as a valuable prognostic factor for screening especially in early stages of disease.

Key word: colorectal cancer, HER2, gene expression

INTRODUCTION

Colon cancer or colorectal cancer is one of the most common cancer in men and women (calvert and frucht.,2002.) . Colon cancer is the third most common type of cancer in 2011, the third leading cause of death is in America. In 2010, about 102 new cases of colon cancer has been diagnosed (sarah popek., etal. 2011) . 5000 new cases of colon cancer are reported annually in iran . According to statistics of the Iranian health Ministry in 1384 reported that about 1130 people died due to this cancer (zhang LQ .,etal.2011.) Colon cancer is the fourth common cancer in the world with an estimated 783,000 new cases per year (Beji A.,etal.2012).

Colon cancer is increasing with rate of 2% annually in the World. Environmental factors and diet are two important factor in 95-85% of cases of colon cancer ( Manmeetkaurgil, et al,2011.).

The maximum of spread of colon carcinoma at the age of 60 to 70 years of age and less than 20% of
cases occur before age 50. In addition to age, lifestyle or environmental factors also are effective in colon cancer. The spread of colorectal cancer are more for men in Japan and Hiroshima and for women in New Zealand. The frequency of colon cancer in men is more than double of in the women. While tumors of the colon happen with equal frequency in both sexes.

Length of survival after diagnosis of colorectal cancer is very different. In America and in many European countries, about 55-50% of patients survive around 5 years. The reason for this difference is unknown and is probably due to the recognition in various stages (Ponz de Leon and Roncucci, 2000).

Several genes are involved in colon cancer, the most important ones are APC, DCC, K-ras, P53, MSH2, MLH1, HER2. In addition to these genes, other genes involved in colon cancer. HER2 is a member of the epidermal growth factor family (EGFR). EGFR with his family Erbb3/HER3, Erbb4/HER4, ErbB2/HER2 make organization homo or heterodimer form.

Through activities such intrinsic tyrosine kinase activation of downstream signaling and it causes phosphorylation of the intracellular domain (Gill MK, et al. 2011). HER2 foment proliferation, migration and invasion in several types of cancer. The levels of protein expression increased when linked (Elsevier, et al. 2014.). HER2 is a membrane glycoprotein that translated from mRNA and located on chromosome 17. Molecular weight of HER2 is 170 kDa.

The oncogene HER2 / neu encodes a membrane tyrosine kinase receptor with extensive homology that linked to epidermal growth factor receptor. HER2 is a gene that sends control signals to the cells to make them grow, divide, and repair guide (Manmeet kaur Gill, et al. 2011).

HER2 is overexpressed in many epithelial malignancies that includes cancers of the pancreas, esophagus, colon and prostate, bladder and sarcoma. In some reports show to have increased by 50 percent of patients with colon cancer. In model of tumors in the colon by increasing HER2 the mitogen and become aggressive form of increased cell motility and invasion and metastasis (Manmeet kaur Gill, 2011.). About 10 to 15 percent of colon cancers are metastatic at diagnosis step and liver is the most common member that include metastasis in colon cancer through the upper rectal veins and take place the transition to this member (Beart, et al. 1983.).

The clinical results in patients being diagnosed in the process of tumorigenesis. Considering the importance of colorectal cancer diagnosed in the early stages to improve treatment and prevent progression of the disease, so diagnosis in the early stages of cancer seems essential (Hong et al., 2012.).

Recent studies show that increased expression of HER2 could be a reliable factor to diagnose the disease at an early stage, or is prognostic factor in colorectal cancer. So far the use of HER2 testing as a screening test for the early detection of determining the risk of colon cancer or early detection in Iran has not been studied and the results of this study can be used in screening or treatment.

MATERIALS AND METHODS

In this study, 40 paraffin tissues (30 patients, 10 normal subjects) were selected for sectioning by apathologist. 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 mM dNTP (CinnaGen, Iran), 0.2 µg of random hexamer (CinnaGen, Iran), 0.5 µg oligo dT (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 HER2 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

<table>
<thead>
<tr>
<th>Amplicon Size</th>
<th>Primer</th>
<th>Name</th>
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<tbody>
<tr>
<td>79 bp</td>
<td>AGACACGGTTGTCCATGCG</td>
<td>HER2=F</td>
</tr>
<tr>
<td></td>
<td>GGTGTAAGGCGAGGGAGTCA</td>
<td>HER2=R</td>
</tr>
<tr>
<td>85 bp</td>
<td>CCCACACACATGCATTACC</td>
<td>GAPDH F</td>
</tr>
<tr>
<td></td>
<td>TGCCTGTCTTCCCTAGCCCT</td>
<td>GAPDH R</td>
</tr>
</tbody>
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**Optimization of real-time PCR essential factors for GAPDH and HER2:**
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 (1x), 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 nonspecific 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 △△Ct. 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 30 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 HER2 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.

![Melting curve analysis of GAPDH and HER2 genes](image)
samples was calculated by the device and the chart was drawn. The results were plotted by using Graphpad software (Figure 2).

**Figure 2:** HER2 gene expression analysis in patients compared with control

As shown in Figure 3, the highest gene expression levels were observed for the sample numbers, 9, 7, 12, 11, 14 all of which were in the third stage. Sample numbers, 13, 17 were in stage 2 and they showed lower expression levels compared with those of the samples in stage 3. Sample numbers, 8, 15, 16, 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 HER2 gene expression was increased by 6/9 times in patients aged less than 50 years compared with that in the normal samples, and it was increased by 7/9 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 HER2 gene expression is directly related to aging.

**Figure 3:** the average amount of HER2 gene expression of patients with 50 years old and above.

Regarding the stages of disease, it was observed that the average increase in HER2 gene expression was 24/2 (P<0/005) in stage 1, 54/1(P<0/005) in stage 2, and 11/35 (P<0/005) in stage 3(Figure 4), which indicates that the expression of HER2 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 HER2 gene can be affected during the disease process and it can be an important candidate factor in the evaluation of cancer.
DISCUSSION
Colon cancer is the third common cancer among men and the second among women in the world. The highest spread of this cancer has been reported in Australia, New Zealand, Europe and North America while the lowest rates are found in Africa and South Central of Asia (Edward et al 2010.). Colon cancer is the third most common cancer and the third leading cause of death in America for men and women. spread of Colon cancer has been fixed at 5% in industrialized nations. Despite its prevalence in developing countries is increasing. Assumes an increase exposure to risk factors (Sarah popek.,etal.2011). Mutations in some genes known as colon cancer mechanisms. The main function of colon cancer genes have been identified in the past decade as well. The proposed three categories of genes involved in colorectal cancer, including tumor suppressor genes, DNA repair genes (Vogelstein and Kinzler, 2004). HER2 gene is oncogenic. A matched Root from HER1 and HER3 and HER4 operate the proliferation, migration and invasion in several types of colon cancer. When HER2 gene linked lead to increasing expression levels (Elsevier.,2014 ). HER2 plays an important role in cell growth and differentiation. The HER2 signaling pathways that trigger the MAPK and P13K is launched routes as a key genes important for cell survival. The important signaling pathways that operate HER2 included trigger the MAPK and P13K pathways and as a key genes are important for cell survival. Increased expression of HER2 guides tumor cell to malignancy.

**Figure 4:** The average amount of HER2 expression in samples with regard to the stages of the disease

**Figure 4:** The average amount of HER2 expression in samples with regard to the stages of the disease.
observation of REAL TIME PCR and IHC .

HER2 is an antigenic marker in immunology studies in colon carcinoma which can be used to in predict and prognosis ( Manmeet okavanagha, etal.2009) . The most common method used to detect the gene HER2 / neu is immunohistochemistry. The results of non-quantifiable, non-repeatability and long time are the major issues of this method That has led researchers try to find new high tech methods based on molecular techniques such as Real Time-PCR.

There are many methods for evaluation HER2 that include immunohistochemistry, ELISA analysis of tumor cytosol, Western blot which indicate methods that HER2 gene is amplified, which is including PCR, FISH, CISH ( King, etal.1984). All these methods have mistaken. There is a possibility of a wrong answer but the Real-Time PCR method is more sensitive than other methods and considered it as a method with high performance and in many research and diagnostic centers is available.Good quantity and quality of RNA extraction for gene analysis method is very important by Real Time PCR . Expensive kit provided with the difficult problem of the use of this valuable resource that their use is very limited. In this study, we set up and optimize manual methods for RNA extraction and Real Time PCR was performed to optimize test although a small step for the localization of the tests carried out in our country and This study for the first time to investigate the increased expression of the HER2 gene in colon cancer patients studied by Real Time PCR and we hope that in the future done studies on larger populations of patients with colon cancer. For a comprehensive view of the accuracy of the test, replace conventional immunohistochemical methods used for diagnosis and treatment. The results showed that the expression of the HER2 gene in cancer patients increased compared to healthy group. So ideas is this factor involved in the development or progression of disease , other results showed that the gene in people over 50 years are over expressed than under 50 years. The results of this study showed that this gene is involved in various stages of disease and according progression and stage of the disease have over expression. Today the role of HER2 has been proven in various cancers such as breast cancer .as targeted treatment for this factor is considered as one of the best ways to control the disease in this type of cancer. The results of this study suggest a role of HER2 in colon cancer. It seems as targeted treatment with a diet therapy for this factor could have been effective to improving in treatment and increased the future chances more.

CONCLUSION

Plans and programs that can be done for screening colon cancer, should be based on the principle detection in the early stages of neoplasms in asymptomatic. Test of HER2 helps to identify process of treatment with AntiHER2. Also an important factor in preventing cancer. As the results of this study demonstrated immunohistochemical method does not confirms method and is only suitable for screening and it must apply a molecular method used to validate the results. The Real time PCR method for reason of high sensitivity and efficiency, can be a valuable alternative or supplementary method instead of immunohistochemistry, as a clinical diagnosis tool. Apply precision molecular techniques such as Real time PCR as a marker for prognosis of HER2 tumor treatment is necessary beside other factors. Due to the significant increase in the amount of HER2 expression in colon cancer samples can be stated that the assessment of HER2 expression as a prognostic factor and valuable, especially in the early stages is considered for screening.

REFERENCES:


