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Comparative Analysis Of Rt-Pcr And Immunohistochemistry Methods For Determining Her2 Status In Breast Cancer Samples

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Abstract
This study aimed to compare two methods of quantitative polymerase chain reaction (qPCR) using the intercalating SYBR Green dye and TaqMan hybridization probes to determine the amount of the HER2 gene (human epidermal receptor) in breast tumours.
Methods--- The experiments were carried out with 32 validated samples of breast cancer and two gastric cancer cell lines. An immunohistochemical (IHC) assay was used to evaluate the accuracy of the RT-PCR methods.
Results--- The obtained results show that real-time PCR with the TaqMan probes allows the use of a small amount of DNA (≥0.4 ng/µl) to determine the overexpression of the HER2 gene. Real-time PCR with SYBR Green allowed us to determine the minimum number of false negatives resulting from the absence of marker expression in tumour tissue.
Conclusions--- The full correspondence of the results of the RTPCR and immunohistochemistry methods obtained for the cell line samples makes it possible to introduce the qPCR method into clinical practice for use in detecting the HER2/neu gene content.

Keywords--- HER2, SYBR Green, TaqMan, breast cancer, PCR, immunohistochemistry

1. INTRODUCTION.

Cancer is most often caused by genetic disorders in cells, in which they undergo uncontrolled growth and proliferation. Cancer is one of the leading causes of death in the world, about 15 million patients with malignant neoplasms are registered annually, and the death rate is more than 9 million people. So, in 2018, 9.6 million people died from this disease [1]. In Uzbekistan, the total number of cancer patients has reached 100,000 [2]. In terms of prevalence, breast cancer is in second place (2.09 million cases) after lung cancer, in terms of death rate it is in fifth place (627,000 cases) [1].
Currently, many research and scientific centers around the world are conducting research to study the determination of the status of HER2/neu in tumor diseases. In recent years, significant advances have been made in studying the expression of the HER2/neu gene at the cellular level, which is due to the development of new modern methods of analysis, such as based on fluorescent *in situ* hybridization (FISH) and chromogenic in situ hybridization

(CISH) [3], as well as immunohistochemical (IHC) [4,5] and PCR analysis [6,7]. Thus, there remains a need for both the study of the biological characteristics of HER2-positive breast tumors and the selection of optimal diagnostic approaches in assessing the status of the HER2/neu oncogene. The development of domestic test systems for assessing the status of HER2/neu seems promising, which is a new science-intensive and resource-saving technology based on modern achievements of fundamental science and developments corresponding to world analogues.

2. MATERIALS AND METHODS.

To develop a method for determining the HER2/neu status based on RT-PCR in breast cancer samples, a series of tests were performed to optimize the PCR conditions. The method is based on the difference in *Ct* amplification cycles of the HER2 and β-globin genes depending on the copy number of the HER2 gene with respect to the reference β-globin gene. To study the quantitative content of the HER2 gene and β-globin in the blood and tumor tissue of breast cancer patients by real-time PCR, primers were selected based on the DNA sequences of genes with a known structure. The result of the research was the development of a working protocol for the quantitative determination of the level of the oncogene HER2 and β-globin based on real-time PCR technologies. The stages of optimization of the PCR conditions were: determination of the concentrations of the cDNA template, oligonucleotide primers, *SYBR Green* and *Taq Man* kits, as well as the selection of denaturation, annealing and DNA synthesis modes.
Determination of the dose of the investigated gene HER2 and the reference gene β-globin in the blood and tumor tissue of patients with breast cancer was carried out in separate wells during one round of RT-PCR.

3. RESULTS.

To optimize RT-PCR using the *SYBR Green* intercalating dye, we used gastric cancer cell lines tested by immunohistochemistry and the results known to us: AGS (*gastric adenocarsinoma*) cell line - HER2 negative and HGE (*human gastric epithelia*) cell line - positive HER2.
As a result of RT-PCR using the *SYBR Green* intercalating dye in DNA samples isolated from both cell lines, the amplification of the HER2 and β-globin genes proceeded at the level from 18 to 25 cycles. The difference between the *SYBR Green* RT-PCR cycles was 0.03 ± 0.0039 on AGS and 5.16 ± 0.044 on HGE (Table-1).
Table-1. Results of quantitative analysis of PCR-RT *SYBR Green* DNA samples isolated from cell lines

Cell Lines	Isolated DNA Concentration	*Ct-SYBRGreen*			HER2 Status
		HER2	*β-globin*	Δ*Ct*	
AGS	124,6	*24.64±0.37*	*24.67 ±0.41*	*0.03 ±0.0039*	HER2 - negative
HGE	81	*18.76±0.44*	*23.92 ±0.45*	*5.16 ±0.044*	HER2 - positive

The high amount of the HER2 gene in comparison with the β-globin gene indicates that the HER2 positive status of tumor cells, as a result of which the amount of the HER2 receptor on the surface of tumor cells increases and, as a consequence, the process of tumorigenesis is triggered. In HER2-negative variants, the difference between the *Ct* of the HER2 and β-globin genes of tumor tissues does not go far from the difference between the cycles of the

HER2 and β-globin genes of blood cells. The experimental results indicate that the optimization has been completed, the threshold values have been determined, and it is necessary to compare the RT-PCR using the *SYBR Green* intercalating dye with other standard methods.

Also, the *SYBR Green* RT-PCR method was used to study 32 DNA samples from breast cancer patients, which had previously been tested for HER2 status by IHC. Patients were aged from 37 to 80, meanage 55 ± 2.

In this study, the amplification of the HER2 and β-globin genes isolated both from the blood and tissue material of breast cancer patients proceeded in parallel at the level of 20 to 33 cycles. HER2-positive was detected in 16 (50%) samples, HER2-negative in 16 samples (50%). The difference between the cycles of HER2-positive samples varied from 4.01 ± 0.053 to 5.2 ± 0.037, for HER2-negative samples - from 0.7 ± 0.058 to 3.75 ± 0.068.

In addition to *SYBR Green* RT-PCR, RT-PCR was optimized using *TaqMan* hybridization probes. To optimize RT-PCR using *TaqMan* hybridization probes, we also used gastric cancer cell lines tested by immunohistochemistry and the results we know: AGS cell line - HER2 negative and HGE cell line - HER2 positive. When RT-PCR was performed using *TaqMan* hybridization probes, the amplification of the HER2 and β-globin genes proceeded at the level of 20 to 34 cycles. The difference between the RT-PCR *TaqMan* cycles was 1.67 ± 0.046 on AGS and 5.61 ± 0.041 on HGE. This gave us the opportunity to conclude that the optimization is complete. (Table 2).

Table-2. Results of quantitative analysis of *TaqMan* RT-PCR of DNA samples isolated from cell lines

Cell lines	IHC result	*Ct- TaqMan*			HER2 status
		HER2	*β-globin*	*ΔCt*	
AGS	0	*29.08±0.42*	*30.75 ±0.49*	*1.67 ±0.046*	HER2 - negative
HGE	+3	*22.94±0.45*	*28,55 ±0.36*	*5,61 ±0.041*	HER2-positive

For these thresholds, it became possible to divide samples into HER2-positive and HER2-negative. This protocol of the RT-PCR method for practical application must be compared with another standardized world-class method.

Also, the *SYBR Green* RT-PCR method was used to analyze HER2 status on 32 DNA samples from breast cancer patients, which had previously been tested for HER2 status by IHC. At the same time, simultaneously with DNA samples from tumor tissues, RT-PCR was performed on blood DNA samples of the same patients. In HER2-positive variants, the amount of HER2 increased significantly, and in the blood it remained almost the same with the amount of the β-globin gene. In the HER2-negative variant, the results of RT-PCR using TaqMan hybridization probes show that, both in DNA samples from tumor tissues and in DNA samples from blood, the amount of the HER2 gene remains almost the same as the amount of the reference β-globin gene.

HER2-positive was detected in 18 (56.25%) samples, HER2-negative in 14 samples (43.75%). The difference between the cycles of HER2-positive samples varied from 5.48 ± 0.047 to 10.87 ± 0.055, in HER2-negative samples - from 0.56 ± 0.018 to 3.5 ± 0.027.

After comparing the results of RT-PCR with the results of the immunohistochemistry method, the threshold values obtained as a result of RT-PCR make it possible to select groups of patients for the use of targeted therapy.

Of all study samples, 32 samples of patients diagnosed with breast cancer had HER2 results determined by immunohistochemistry. 14 (43.75%) of them had an overexpression of HER2

under an indicator of +3, 6 (18.75%) patients had an indicator of +2 immunohistochemical method, which is controversial, depending on the intensity of staining and the experience of a specialist. In 12 (37.5%) of the patients, no overexpression of HER2 was detected, 8 (25%) of them under the index +1 and 4 (12.5%) patients with a diagnosis of breast cancer under the index 0 according to the results of the immunohistochemical method (Table 3).

Table-3.Immunohistochemistry results for HER2 status in patients with breast cancer

HER2 status	Immunohistochemistry parameters				
	Score 0	Score +1	Score +2	Score +3	total
HER2 quantity	4	8	6	14	32

Using these parameters, a comparative analysis of the results of RT-PCR performed using the *SYBR Green* intercalating dye and *TaqMan* hybridization probes with the results of immunohistochemistry was carried out.

As a result of RT-PCR using the *SYBR Green* intercalation dye and *TaqMan* hybridization probes in DNA samples isolated from both cell lines, amplifications of the HER2 and β-globin genes proceeded at the level of 18 to 31 cycles. The difference between the *SYBR Green* RT-PCR cycles was 0.03 ± 0.0039 on AGS, 5.16 ± 0.044 on HGE, and *TaqMan* RT-PCR, respectively, 1.67 ± 0.046 and 5.61 ± 0.041. This difference in *Ct* makes it possible to conclude that the results of PCR and IHC are 100% the same. (Table 4)

Table-4. Comparison of the results of IHC and RT-PCR methods for the status of HER2 cell lines

Cell Lines	IHC Result	*Ct-SYBRGreen*			*Ct-TaqMan*			HER2 Status
		HER2	*β-globin*	Δ*Ct*	*HER2*	*β-globin*	Δ*C*	
AGS	0	*24.64 ±0.37*	*24.67 ±0.41*	*0.03 ±0.0039*	*29.08 ±0.42*	*30.75 ±0.49*	*1.67 ±0.046*	HER2 negative
HGE	+3	*18.76 ±0.44*	*23.92 ±0.45*	*5.16 ±0.044*	*22.94 ±0.45*	*28,55 ±0.36*	*5,61 ±0.041*	HER2 positive

Studies by Kurabayshi and others show that according to color and number of receptors, IHC results are subdivided into (0), (+1), (+2), (+3). With (0), (+1), the HER2 status is definitely considered HER2-negative. When (+2), based on the intensity of the staining and the experience of the specialist, it can be either HER2-negative or HER2-positive. The result (+3) means the positive status of the oncogene. Comparison of test results by IHC and PCR methods was carried out in 32 samples of breast tumor tissue, the summary results of which are presented in Table 5.

Table-5. Comparison of the results of assessing the status of HER2 by RT-PCR and IHC

PCR results	IHC result									
	Score 0		Score +1		Score +2		Score +3		итого	
	4		8		6		14		32	
	PCR SYBR Green	PCR TaqMan	PCR SYBR Green	PCR TaqMan	PCR SYBR Green	PCR TaqMan	PCR SYBR Green	PCR TaqMan	PCR SYBR Green	PCR TaqMan

HER2-positive					4	6	12	12	16	18
HER2 negative	4	4	8	8	2		2	2	16	14
Bcero	**4**	**4**	**8**	**8**	**6**	**6**	**14**	**14**	**32**	**32**

These results show that the *SYBR Green* RT-PCR and *TaqMan* RT-PCR are fully consistent with the IHC results. Under the indicator (+2) by the IHC method, there are 6 samples, by *SYBR Green* PCR 4 of them are HER2-positive, two are HER2-negative. All 6 samples come out HER2-positive by *TaqMan* PCR protocol. These results indicate that the difference between the *SYBR Green* RT-PCR and *TaqMan* RT-PCR protocols is 6.25% (Figure 1).

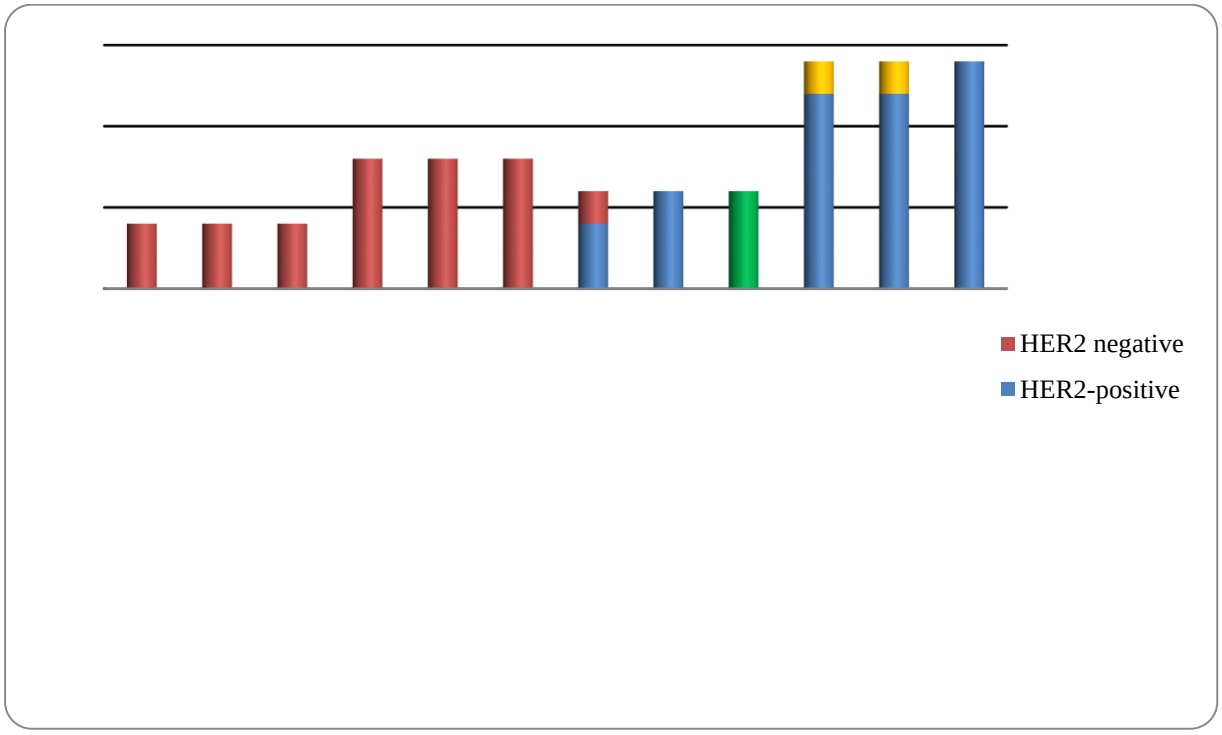

Figure-1. Comparison of the results of assessing the status of HER2 by RT-PCR and IHC

4. DISCUSSION.

In this study, the comparison of the results of IHC and RT-PCR using the *SYBR Green* intercalation dye corresponds to 87.5%, and for real-time PCR with *TaqMan* hybridization probes corresponds to 93.75%. The discrepancy with the results of IHC can be explained by the fact that an increase in the amount of the gene may not be observed at the DNA levels, and the increased level of proteins makes it possible to assess the status of HER2 by IHC. Also, the discrepancy in the results may be due to the lack of specificity of the IHC method, in which the total content of the HER2/neu receptor proteins is determined. Nevertheless, in most cases, RT-PCR showed coincidence with IHC.

The results obtained show that the recorded increase in the dose of the HER2/neu gene depends on the method used. *TaqMan* RT-PCR allows to work with a small amount of DNA to determine the overexpression of the HER2 gene, because, the fact that the added hybridization probes that are used with the *TaqMan* set of mixtures increase the sensitivity, and therefore the difference in *Ct* values between HER2 and β-globin increases. In some cases, the *SYBR Green* technology has led to an underestimation of the obtained data, which significantly limits the use of this technology in the analysis of the increase in the dose of the HER2 / neu gene in cancer. This confirms that RT-PCR TaqMan is a method that allows one

to obtain a minimum number of false negative conclusions about the absence of an expression marker in tumor tissue.

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