Research Article

A Novel miRNA Located in the HER2 Gene Shows an Inhibitory Effect on Wnt Signaling and Cell Cycle Progression

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Human epidermal growth factor receptor 2 (HER2), frequently called HER2 receptor tyrosine kinase 2, is the most oncogenic member of the human epidermal growth factor (EGFR) family [1, 2]. Often, the HER2 receptor indirectly binds to its ligands and forms heterodimers with other EGFR receptors, preferentially with ErbB3 [3, 4]. Dimers containing HER2 can activate a variety of signaling pathways such as MAPK, protein kinase C (PKC), and AKT pathways [5]. Based on the cellular context and HER2 partner, a wide range of cellular responses could be assigned by following HER2 activation, including proliferation, migration, adhesion, differentiation, and apoptosis. Furthermore, heterodimers prolong downstream signaling activation and increase their outputs in comparison with EGFR homodimer members [6, 7].

MicroRNAs (miRNAs) are a group of noncoding RNAs with ~ 22 nucleotides long that control numerous cellular process like survival, apoptosis, differentiation, and tumorigenesis. miRNAs can bind to the 3′-UTR segment of mRNAs and adjust the expression of their targets via either mRNA degradation or interference in protein translation [8–11]. Lin4 and let7 as the first miRNAs were discovered in C. elegans using forward genetics methods [12]. However, due to the small sizes of miRNAs, and their susceptibility to mutations, the identification of novel miRNAs using forward genetics approaches is very difficult [13, 14]. On the other hand, several bioinformatics tools are developed to find human miRNA hairpin structures. These software programs are developed to find the main criteria of miRNAs including the stem-loop structure of miRNA precursors, phylogenetic conservation, and thermodynamic stability of stem-loops as well as the genomic location of validated miRNAs [10, 14]. About 55000 bona fide miRNA genes have been guessed to be in the human genome, and ~2500 human mature microRNAs have been registered in the miRBase database (http://www.mirbase.org/) to date [15, 16]. Following our previous successful bioinformatics prediction and
2. Materials and Methods

2.1. Bioinformatics Prediction. Using SSC profiler (http://mirna.imbb.forth.gr/SSCprofiler.html) [19], MiPRED (http://www.bioinf.seu.edu.cn/miRNA) [20], and Mireval (https://omic.tools.com/mireval/) [21] online tools, HER2 gene was scanned to find possible stem-loop structures, potentially encoding novel miRNAs. A potential hairpin structure located in the 5th intron of HER2 gene had all the features of the bona fide miRNA precursor, and the production of a mature miRNA was verified experimentally. Overexpression of the implied miRNA produced expected phenotypes, such as the alteration of c-Myc, APC1, and APC2 gene expression and also suppression of the Wnt signaling pathway.

2.4. DNA Constructs. To clone the DNA of the predicted pre-
HER2-miR1, a piece of the human HER2 gene, about 300 bp, was PCR amplified using Int-5-F and Int-5-R primers (Table 1). This amplicon was cloned in the pEGFP-C1 expression vector (Clontech) downstream of the GFP gene. A sequence forming the hairpin structure that was cloned earlier into the pEGFP-C1 vector [8] was used as a scrambled negative control. All of these recombinant constructs were isolated and sequenced for confirmation of the right inserts.

2.5. Overexpression and Knockdown of HER2-miR1 in Cell Lines. The studied cell lines which were cultured in 24 well plates were transfected with two micrograms of recombinant pEGFP-C1 vector containing HER2-miR1 precursor which were covered by Lipofectamine 2000 (Invitrogen). Mock and scrambled vectors were used as negative controls. After twenty-four hours of transfection, GFP expression was assessed with an invert fluorescence microscope (Nikon eclipse Te2000-s).

2.6. Analysis of the Cell Cycle. Cells were transfected with a HER2-miR1 overexpression cassette and after 36 hours of transfection were harvested and dyed with propidium iodide (PI) (Roche). Triton X100 and RNase A were used to make the cells PI permeable and remove the cell’s RNA, respectively. All assays were carried out using a FACS flow cytometer and analyzed with Cell Quest software (BD Biosciences).

2.7. Detection of HER2-miR1. RNA was extracted from HEK293t cells forty-eight hours after transfection with a pre-
HER2-miR1 overexpression cassette. Then, 3'-Poly-A tail was added to RNAs in a reaction containing 5 U Poly-A polymerase (Biolabs), 4 μl 10 mMol ATP and 2 μg of extracted RNA. According to our previously described protocol, the first-strand cDNA was synthesized using Rever-seAid Reverse Transcriptase (Thermo Science) utilizing the specific anchored-oligo-d'T primers [10]. Real-time quantitative PCR (RT-qPCR) was performed in an ABI PRISM 7500 system (Applied Biosystems), according to the following run method: the initial denaturation 15 min at 95°C and 48 cycles of 95°C/15 s, 65°C/20 s, and 75°C/15 s. RT-qPCR was performed according to MIQE guidelines using EvaGreen master mix (Amplicon). GAPDH and U48 small nucleolar RNA (SNORD48) were used as reference genes [29, 30]. The RT-qPCR data were analyzed using the 2^-ΔΔCt method [31].

2.8. TOP/FOPflash Assay. TOP/FOP reporter assays were performed with Dual-Glo Luciferase Kit based on the manufacturer’s instructions (Promega). In brief, the SW480 cells were transiently cotransfected with TOP or FOPflash constructs (1 μg) and preHER2-miR1 construct and also scrambled and mock negative vector (1 μg) in triplicate. TOPflash was assayed forty-eight hours after transfection for each vector.

2.9. Statistical Analysis. The GraphPad Prism 5.04 (GraphPad, San Diego, CA, USA) was used for the statistical analysis. Flow cytometry results (PI test) were interpreted with
flowing software 2.5.1 (Flowing software, Turku, Finland). In all analyses, P value < 0.05 was considered statistically significant. Each experiment was performed in duplicate, and the assays were replicated at least two times. The data that support the findings of this study are available from the corresponding author upon reasonable request.

3. Result

3.1. Prediction of a Novel miRNA within the Intron of the Human HER2 Gene. SSC profiler program was used to predict possible stem-loop structures within the HER2 gene (Figure 1(a)). This program demonstrated about 80 stem-loops within its exons and introns. One of these stem-loops, hg17, chr17: 35109679-35109728 that here we named pre-HER2-miR1 (Figure 1(b)), had most of the criteria for producing a real mature miRNA, named HER2-miR1. miR-FIND, mature Pred, and MatureBayes along with MiPRED recognized pre-HER2-miR1 as a real miRNA precursor with a significant score. Moreover, the UCSC Genome Browser blast search illustrated that the HER2-miR1 and its precursor are conserved in mammals (Figure 1(d)). Based on the Mireval online tool, HER2-miR1 precursor is strongly conserved and is not homologous with other validated miRNAs. Alignment of HER2-miR1 to mature miRNAs which are registered showed only weak similarity to hsa-miR-4687-3p and bra-miR-164e-3p.

3.2. Detection of Exogenous HER2-miR1 in HEK293t Cell Line. In an attempt to detect mature HER2-miR1, the pre-HER2-miR1 construct was overexpressed in HEK293t cell line. The pEGFP-C1 empty vector (mock vector) and untransfected cells were used as negative controls. The efficiency of transfection was estimated based on visual observation of GFP emission, and the best transfected cells were then selected for RNA extraction (Figure 2(a)). The RT-qPCR analysis showed that for the cells overexpressing pre-HER2-miR1, the HER2-miR1 expression level was increased about 4,000 folds compared to the cells in which the mock vector was overexpressed (Figure 2(b)). Gel electrophoresis proved the right size for the amplification products of exogenous HER2-miR1 by RT-qPCR (Figure 2(c)). When RT-qPCR products with the expected size (about 80 bps) were sequenced (Figure 2(d)), the result revealed the efficient production of mature HER2-miR1. The minimum size of this sequence was submitted to EMBL-EBI database under the accession number # PRJEB10344.

3.3. Detection of Endogenous HER2-miR1 in Various Human Cell Lines. To examine whether the HER2-miR1 is expressed endogenously, RNA samples were extracted from various cell types, including 5637, Hela, NT2, KYSE, A172, HUH7, SW480, HT29, and HCT116 cell lines. Then, HER2-miR1 was especially amplified in the cDNAs prepared from the mentioned RNA extracts, using RT-qPCR. Our results showed that the 5637 cells had the highest level of HER2-miR1. On the other hand, the NT2 and HeLa cell lines had a moderate level of HER2-miR1, while no significant expression was observed for this novel miRNA in other tested cell lines (Figure 3).

3.4. Analysis of HER2-miR1 Direct Interactions with Predicted Target Genes. RNAhybrid tool predicted Axin1 and Akt2 genes as putative direct targets for HER2-miR1 showing one MRE for each gene (Figure 4). Then, following the overexpression of HER2-miR1 and Anti-HER2-miR1, RT-qPCR data confirmed its elevated expression level up to ~30 and ~25 folds, respectively, 48 h after transfection of

| Primer name | Primer sequence 5′ to 3′ |
|-------------|--------------------------|
| HER2-miR1   | GTTTGAGGGGGGCGAGCT       |
| U48         | TGGGCTGTGGGCGAGCT        |
| Anchored oligo-dT | GCGTCGACTAGTGACATCACGAGTTCTCCAGCATCAGGACGAGCAG(T)18 N |
| Universal outer | AACTCAAGTGTCCTCCAGTCAG |
| Universal inner | GCGTCGACTAGTGACATCACGAG |
| GAPDH       | Forward: GTGAACTGAGAAGGATATGA |
| APCI-real time | Reverse: CATGAGTCTCCGAGTAC |
| APCI2-real time | Forward: TATTACGGAATTGTCGCCAGCTTG |
| APCI2-real time | Reverse: CCACATGATTGACTATTGTC |
| c-myc-real time | Forward: CTCCTAGTGGCGTCACAC |
| Axin1-real time | Reverse: CGGCGTGAGCAGTAAACCT |
| Axin1-3′ UTR | Forward: ATGCGAGGAGGGCAGGAGTC |
| Akt2-3′ UTR | Reverse: AAGGTGGAGGGCAGGAGTC |
| GAPDH       | Forward: GTGAACCATGAGAAGTATGA |
| APC1-forward | Reverse: CATGAGTCTCCGAGTAC |
| APC1-forward | Reverse: CCACATGATTGACTATTGTC |
| APC2-forward | Forward: CTCCTAGTGGCGTCACAC |
| APC2-forward | Reverse: CGGCGTGAGCAGTAAACCT |
| Axiin1-forward | Forward: ATGCGAGGAGGGCAGGAGTC |
| Axiin1-forward | Reverse: AAGGTGGAGGGCAGGAGTC |
| Axiin1-forward | Forward: AAGGTGGAGGGCAGGAGTC |
| Axiin1-forward | Reverse: AAGGTGGAGGGCAGGAGTC |
| Akt2-forward | Forward: CTCCTAGTGGCGTCACAC |
| Akt2-forward | Reverse: CGGCGTGAGCAGTAAACCT |
| Akt2-forward | Reverse: AAGGTGGAGGGCAGGAGTC |
SW480 cell line. Also, data showed an elevate expression of HER2-miR1 and anti-HER2-miR1 cassette up to \~40 and \~30 folds, respectively, 48 h after transfection of HEK293T cell line. The intrinsic expression of HER2-miR1 was reduced by \~half, after transfection with anti-HER2-miR1 cassette in both cell lines (Figure 4(a)). RT-qPCR against Axin1 predicted target genes indicated significant expression alteration of this gene at the RNA level in SW480 and HEK293T cells. In overexpressed HER2-miR1 cells, the Axin1 expression showed a significant decrease (\~0.5 and \~0.25 fold) in both cell lines in compassion with overexpressed mock cells. Also, in both cell lines, knowing down of HER2-miR1 by anti-HER2-miR1 cassette caused an about twofold increase in Axin1 expression (Figure 4(a)). But, the AKT2 expression, as one of predicted targets, showed no significant alternation.

3.5. HER2-miR1 Overexpression Effect on the Wnt Signaling Pathway. To analyze the influence of HER2-miR1 on the Wnt signaling pathway, the pGL4-TOP and HER2-miR1 overexpressing vectors were transiently cotransfected into the SW480 cell line (Wnt+). In the control experiment, the HER2-miR1 overexpressing vector was replaced with the mock or scrambled vectors. The elevated level of mature HER2-miR1 in the SW480 cells transfixed with the overexpression cassette of HER2-miR1 was confirmed using RT-qPCR (Figure 5(a)).

To further investigate the influence of HER2-miR1 overexpression on this pathway, three downstream genes were also measured with RT-qPCR in SW480 cells. The APC1 and APC2 gene expression levels were significantly upregulated after HER2-miR1 overexpression in these cells (Figure 5(c)). However, the expression level of the c-Myc gene was significantly decreased in the cells overexpressing HER2-miR1 (Figure 5(c)). To confirm this result, we used two small molecules, PNU-74654 and XAV-939, that inhibit the Wnt signaling pathway [17, 32]. SW480 cells were treated with these small molecules for 10 hours and then transfected with the pre-HER2-miR1 construct. In both cases, the overexpression of HER2-miR1 significantly upregulated APC1 and APC2 genes, whereas c-Myc expression level showed a reduction in the presence of these small molecules (Figure 5(d)).

**Figure 1:** Bioinformatics prediction of preHER2-mir1 within the 5th intron of human HER2 gene. (a) Schematic presentation of HER2 gene adapted from Ensemble. Exons and introns are shown with a rectangular shape and broken lines, respectively. (b) Shows predicted stem-loop encoding HER2-miR1. The red-colored sequence is predicted by SSC profiler as possible HER2-miR1 mature form. (c) Illustrates Drosha cutting sites on the sequence of HER2-miR1 stem-loop predicted by miRFIND tool. (d) Blat search result by UCSC Genome Browser shows strong conservation of HER2-miR1 among several organisms including mammals.
3.6. Cell Cycle Analysis. SW480 and HCT116 (Wnt⁺) and HEK293t (Wnt⁻) cell lines were transfected with the vector overexpressing HER2-miR1. Then, the transfected cells were stained with PI and analyzed with the flow cytometer, 36 h after transfection. About 15% elevation of G1 and ~3.3% reduction of S phases were observed in SW480 cells following the HER2-miR1 overexpression compared with the related controls (Figure 6(a)). Similarly, about 5% elevation of G1 and 4% reduction of S phases were perceived in HCT116 cells overexpressing HER2-miR1 (Figure 6(b)). However, the overexpression of HER2-miR1 did not alter the cell cycle of the HEK293t cell line compared to mock control, significantly (Figure 6(c)).

4. Discussion

MicroRNAs are key regulators of many biological processes including proliferation, differentiation, and apoptosis. Much attention is devoted to the miRNA detection, biogenesis, and function over the last decade, with the goal of novel pharmacological therapy [33]. Methods that rely on computational tools have accelerated the prediction of new miRNAs and their target genes [34]. miRNAs have a small size, have low expression level in tissues and cells, and show time-dependent expression. Therefore, forward genetics has been inefficient in miRNA gene detection [35]. Today, there are several miRNA prediction software programs that accelerate novel miRNA detection [34, 36].

SSC profiler predicted more than 80 stem-loop structures in the human HER2 gene. One of these stem-loop structures that we called it HER2-miR1 and located in its

Figure 2: Production of exogenous HER2-miR1 mature form from its precursor. (a) Fluorescent image (×40) of HEK293t cells transfected with mock vector or the expression vector that carries HER2-miR1 precursor. (b) RT-qPCR results indicated the overexpression of HER2-miR1 (~×4,000 folds) in the HEK293t cells that were transfected in (a). Data were normalized against U48, and error bars indicate standard deviation (SD) of duplicated experiments. (c) Electrophoresis gel image demonstrated the appropriate size of mature HER2-miR1 in the cells overexpressing preHER2-miR1 in comparison with un-transfected cells. (d) Sequencing result of two TA vector clones (named clones 1 and 2) containing HER2-miR1, which is aligned with preHER2-miR1. 3’-end of HER2-miR1 was at least three nucleotides longer than the specific primer shown by a black arrow which was used for amplification of this miRNA. The downstream and upstream nucleotides to HER2-miR1 sequence belong to the vector and anchored oligo-dT primer.

Figure 3: Detection of HER2-miR1 in various cell lines. The maximum expression level of HER2-miR1 was detected in 5637 cells, and the minimum was detected in colorectal cancer-originated cell lines, SW480 and HT29. U48 RNA was used for normalizing the expression levels of HER2-miR1. Error bars reveal the SD of duplicated experiments.
Figure 4: Direct interaction of HER2-miR1 with its predicted target genes. (a) RT-qPCR indicated that HER2-miR1 alteration expression had significant effect on Axin1 and but not Akt2 genes at the RNA level in SW480 and HEK293T cells. (b) Shows the pairing status of HER2-miR1 with predicted MREs in the 3′-UTR sequences of predicted target genes.

Figure 5: Involvement of HER2-miR1 in Wnt signaling pathway regulation. (a) Accumulation of HER2-miR1 in SW480 cells overexpressing preHER2-miR1. (b) Shows significant Wnt signaling downregulation following HER2-miR1 overexpression. Relative luciferase activity was measured in SW480 cells which were cotransfected with TOP-flash vector along with preHER2-miR1 overexpressing vector or mock and scrambled control vectors. Error bars indicate SD of triplicated experiments, $P < 0.05$. (c) Shows the upregulation of the APC1 and APC2 genes and downregulation of c-Myc, following HER2-miR1 overexpression in SW480 cell line. Error bars indicate SD of duplicated experiments, $P < 0.05$. (d) Shows HER2-miR1 overexpression along with PNU-4654 and XAV-939 small molecules effects on APC1, APC2, and c-Myc gene expression. Error bars indicate SD of duplicated experiments, $P < 0.05$. 

Wnt signaling pathway gene expression

HER2-miR1 expression

TOP/FOP flash assay
5th intron (Figure 1(a)) had the most features for producing a bona fide miRNA (Figure 1(b)). Moreover, the miRFIND online tool prognosticated a Drosha processing site in this sequence (Figure 1(c)). Similar to most identified miRNAs [8, 10, 11, 37, 38], UCSC tool illustrated a high conservation pattern for preHER2-miR1 and its mature form in several organisms including mammalians (Figure 1(d)). When high-score target genes, predicted by DIANA MR-MicroT, were categorized by DAVID online tool, it was suggested that HER2-miR1 might regulate the Wnt signaling pathway. In addition, RNAhybrid predicted strong and poor complementation for Axin1 and Akt2 as the target genes for HER2-miR1, respectively (Figure 4(b)). Generally, all of these employed bioinformatics software programs strongly supported the presence of this novel miRNA in the HER2 gene.

Protein Atlas [28] and EMBL-EBI databases indicated that the HER2 gene (and probably HER2-miR1) is slightly expressed in HEK293t cell line, which is efficiently transfected [39]. Then, preHER2-miR1 was overexpressed in these cells, and exogenous mature HER2-miR1 was detected using the reported approach [8, 10] (Figure 2(a)). RT-qPCR amplification products of HER2-miR1 overexpression (Figure 2(b)) with the expected size (Figure 2(c)) were cloned and sequenced. Two colonies had the same nucleotide sequences and well-matched to preHER2-miR1. Micro-RNAs are described to be 18-27 nucleotides long [8, 10, 11, 40, 41]; here, HER2-miR1 was at least 20 nucleotides long (Figure 2(d)).

Endogenous detection of a predicted novel miRNA is supportive evidence for its identity [14]. The cell lines originated from colorectal cancer, including HCT116, HT29, and SW480, presented the lowest level of Her2-miR1 expression. It is consistent with the very low expression level of the HER2 gene in SW480 that is reported elsewhere [42]. On the other hand, the bladder-originated 5637 cells, which express HER2, showed the highest expression level of HER2-miR1.

Figure 6: HER2-miR1 overexpression effect on cell cycle status of Wnt+ and Wnt- cells. (a, b) Show HER2-miR1 overexpression effect on SW480 (a) and HCT116 (b) cell cycle distribution, 36 h after transfection, measured by PI. Overexpression of HER2-miR1 resulted in significant G1 elevation and S phase reduction in both SW480 and HCT116 cell lines, compared to the related controls. (c) Overexpression of HER2-miR1 did not significantly change the cell cycle population distribution of HEK293t cell line. Error bars indicate SD of duplicated experiments, $P < 0.05$.
(Figure 3) [43]. Relatively higher expression of HER2-miR1 in 5637 cell line suggests that this miRNA may be useful for further analysis in bladder cancer specimens as a diagnostic biomarker.

Hence, HER2-miR1 was successfully overexpressed in SW480 (as a Wnt +) cell line (Figure 5(a)), and the Wnt pathway activity was evaluated using TOP/FOPflash assay system (Figure 5(b)). Our results showed that HER2-miR1 downregulated the Wnt pathway compared with scrambled and mock controls (Figure 5(b)). Consistent with the down-regulation of the Wnt signaling pathway (Figure 5(b)) and down-regulation of c-Myc (Figure 5(c)) as a known downstream gene for the Wnt signaling (51), APC1 and APC2 genes as Wnt signaling inhibitors [44, 45] were upregulated, following the HER2-miR1 overexpression (Figure 5(c)). Also, HER2-miR1 showed synergic inhibitory effects on the Wnt signaling with XAV-939 and PNU-74654 small molecule (Figure 5(d)). Overall, these results suggest that HER2-miR1 may play a role as a negative regulator of the Wnt signaling pathway.

Consistently, overexpression of HER2-miR1 in both SW480 and HT29 as Wnt + cells [46] resulted in the increased and decreased proportion of the cells at the G1 and S phase in transfected cells, respectively (Figures 6(a) and 6(b)). Such an effect was not detected in Wnt- HEK293t cells (Figure 6(c)) [47]. These controversial cell cycle effects of HER2-miR1 could be attributed to differences between physiological and cellular conditions and genetics and epigenetics backgrounds of the studied cell lines. In conclusion, here, we have presented HER2-miR1 as a novel conserved miRNA mapped within the 5th intron of the human HER2 gene and provided pieces of evidence concerning its specifications and functionality in the adjustment of the Wnt signaling pathway, especially by upregulating APC1 and APC2 gene expression. Considering the role of HER2 signaling in breast cancers, it has remained to be tested if HER2-miR1 affects breast cancer initiation and progression.

Data Availability
No data were used to support this study.

Ethical Approval
This article does not include any experiments with human participants or animals carried out by any of the authors.

Conflicts of Interest
The authors declare that there are no conflicts of interest with any financial organization regarding the material discussed in the manuscript.

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