Immunogenicity Evaluation of Recombinant Edible Vaccine Candidate Containing HER2-MUC1 against Breast Cancer

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ABSTRACT

Human epithelial growth factor receptor2 (Her2) and polymorphic epithelial mucin (MUC1) are tumor-associated antigens that have been extensively investigated in adenocarcinomas. Generally, each of these molecules was used separately for diagnosis of adenocarcinomas and injective vaccine therapy researches, but not in the chimeric form as and edible immunogen.

In this study, Her2, MUC1, and a novel fusion structure were expressed in the seeds and hairy roots of transgenic plants appropriately. The mice groups were immunized either by feeding of transgenic seeds or hairy roots.

All immunized groups showed a considerable rise in anti-glycoprotein serum, IgG and IgA, and IFNγ cytokine. However, the animals received chimeric protein showed significant higher immune responses in comparison to ones received these immunogen separately.

The results indicated that the oral immunization of an animal model with transgenic plants could effectively elicit immune responses against two major tumor-associated antigens.

Keywords: Breast cancer; Edible; HER2; Immunogen; MUC1; Transgenic plant

INTRODUCTION

Human epithelial growth factor receptor 2 (Her2) has been found to play a remarkable role in a number of the most common human solid tumors including but not limited to breast, ovarian, endometrial, colon, non-small cell lung cancer, prostate, and cervical cancer as well as to lupus nephritis.1,2 Approximately, in 30% of patients with breast cancer, the Her2 gene is amplified and its related receptor is overexpressed on the tumor
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cell surface. This molecule plays a crucial role in the malignant transformation and aggressiveness of breast cancer.\(^5\) The human mucin-like molecule, polymorphic epithelial mucin (MUC1), a tumor-associated antigen, is a polymorphic transmembrane glycoprotein expressed on the apical surface of glandular and simple epithelial cells.\(^4\) MUC1 is excessively overexpressed and aberrantly O-glycosylated in a wide variety of adenocarcinomas. The extracellular domain of mucin containing a variable number of 25–125 tandem repeats of 20 amino acids.\(^7\) These qualitative and quantitative changes of MUC1 expression in cancer render it immunogenic. Some studies have identified anti-MUC1 immune responses in preclinical investigations and with cancer patients after active immunization with MUC1.\(^6,7\)

For the production of transgenic Her2 and MUC1 polypeptides, selecting a suitable host is important. Among different heterologous hosts for recombinant protein production, the plants can work as outstanding platforms because of their high capacity and fast scaling-up which can decrease costs.\(^8\) It is proved that the human recombinant proteins produced in plants can be properly assembled and folded in their similar structure, as in humans.\(^9,10\)

In principle, plant-based vaccines can induce mucosal immunity effectively. The plant cells act as a natural microencapsulation system to prevent the vaccine antigens from being degraded in the upper digestive tract before they reach the gut-associated lymphoid tissue.\(^11\) Transgenic hairy roots can be used for producing recombinant proteins with the biosafety consideration such as lack of pollination and control of gene transfer.\(^12\) The seeds have the appropriate biochemical environment such as high protein content, low protease, and water activities\(^13\) which could promote stable protein accumulation.\(^14\) Many recombinant proteins have been produced in some agronomic plant species such as tobacco, canola, and maize successfully.\(^15\) Canola seeds with its high protein content (20% w/w) can be modified to produce high levels of recombinant heterologous protein for more efficient delivery of an oral immunogen.\(^16\) In the previous studies, polypeptides from different parts of breast cancer antigens have been produced successfully in plant systems, injected into animal models and stimulated the immune response.\(^17-19\) However, it seems that chimeric protein which consists of two important breast cancer antigens could be more effective to defeat the disease. According to our literature review, there have been no studies performed on edible Her2 or MUC1 or HM (Her2-MUC1) immunogens. In some cases, it was shown that recombinant plant-made proteins can be used without any protein extraction process to reduce the cost more than eighty percent.\(^20,21\)

In this regard, and based on our previous insilico it is a correct prediction, we expected that our designed recombinant immunogens can be effectively produced by plant cells, present all the necessary modifications resembling human cells, and stimulate the immune system of treated mice effectively.

**MATERIALS AND METHODS**

**Chimeric Gene Design**

The chimeric antigenic construct HER2-MUC1(HM with Acc. no. KF443783) consisted of the C-terminal (480-620) of HER2 and seven repeats (VNTR) which were analyzed by bioinformatics softwares.\(^8\) The multimeric gene was synthesized by ShineGene (China).

**Construction of Plant Expression Vectors**

The HM gene was subcloned in Cfr9II/Sacl sites of pBI1400\(^22\) and in Xbal/Sacl sites of the pBI121 vector (Novagen, USA). In both vectors, the \(\beta\)-glucuronidase gene was replaced with the synthetic HM gene. The resulting plasmids (pBI1400-HM and pBI121-HM) were utilized for the transformation of B. Napus (Brassica napus) and N. Tabacum (Nicotiana tabacum), respectively. The recombinant authentic plasmids were separately introduced into Agrobacterium tumefaciens LBA4404 cells.\(^23\) The recombinant A. tumefaciens cells were verified by PCR analysis and used for plant transformation. The separate genes encoding HER2 and MUC1 were amplified from the original plasmid by standard PCR using Pfu DNA polymerase. Sequencing and digestion were performed to evaluate the integrity of these four (pBI1400 or pBI121 with HER2 or MUC1) constructs.

**Plant Transformation and Regeneration**

Commercial canola (B. napus L, CV. PF 7045-91) and CV. Samsun (N. tabacum) seeds were obtained from the Seed and Plant Improvement Research Institute of Iran. The transformation of both plants was carried out using the methods described previously.\(^23\)
For the production of transgenic hairy roots, the plants germinated from T1 seeds of the transgenic *N. tabacum* were infected by immersing into an *Agrobacterium rhizogenes* ATCC 15834 suspension culture. The hairy roots appeared after ten days and were cultured in liquid MS medium without any additive.

**PCR Analysis of Transgenic Plants**

The presence of transgenes was determined by PCR analysis on genomic DNA using specific primers (Table 1 and Figure 1). The attachment sites of the primers and restriction sites of restriction enzymes are shown in Figure 2.

![PCR Analysis of Transgenic Plants](image)

**Figure 1.** Molecular analysis of transgenic plants. A) Amplification of *HM* (A1, 1051bp) and Her2 genes (A2, 653bp) in transgenic *B. napus* with the specific FAE (Fatty Acid Elongase) /NOS primers and MUC1 gene (A3, 518bp) in transgenic *B. napus* with the specific MUC1 primers. B) Amplification of *HM* (B1, 1017bp) with the specific Her2/MUC1 primers and Her2 genes (B2, 477bp) specific Her2 primes and MUC1 gene (B3, 518bp) with the specific MUC1 primers in transgenic *N. tabacum*. In all gels lanes 1–4: Transformed plant lines expect in the B1 gel which lanes 1-2 are transformed plant lines, (-): Non transformed plant and Lane M: Gene Ruler DNA Ladder Mix (Thermo Fisher Scientific). (+): pBI1400-HM plasmid in A1 and B1, pBI1400-Her2 plasmid in A2 and B2 and pBI1400-MUC1 plasmid in A3 and B3 (The gels are slightly cropped for improving the clarity and conciseness of the presentation).

![Schematic representation of pBI1400-HM/pBI121-HIM constructs](image)

**Figure 2.** Schematic representation of pBI1400-HM/pBI121-HIM constructs. The constructs contain the HM gene consisting of coding sequences for the immuno-protective epitopes of the HER2 and MUC1 antigens under the control of the promoter (either FAE or CaMV35S (Cauliflower Mosaic Virus 35S)). The primers’ attachment sites are shown with arrows.
Table 1. The primers’ names, sequences and products sizes (it is clear, no need to more explanation.)

<table>
<thead>
<tr>
<th>primers’ names</th>
<th>products sizes (bp)</th>
<th>Tm (°C)</th>
<th>primers’ sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Her2 forward primer (HeF)</td>
<td>465</td>
<td>60</td>
<td>AGATCTCCCCGGGTCTAGAAAAACAATGGCTCCTTTG</td>
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<tr>
<td>Her2 revers primer (HeR)</td>
<td></td>
<td></td>
<td>TCAGCGAGCTCTAGTTACGTAGTTTCCTCTGAGAACTTTCC</td>
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<tr>
<td>MUC1 forward primer (MUF)</td>
<td>486</td>
<td>56</td>
<td>AGGCAACCCCAGGTCTAGAAAAACAATGGCTCCTGACGAGCCTG</td>
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<tr>
<td>MUC1 revers primer (MUR)</td>
<td></td>
<td></td>
<td>CAAATTTGAGATCGGAGATCAGTCTCAGTCTCAGTCTG</td>
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<tr>
<td>actin forward primer (acF)</td>
<td>380</td>
<td>57</td>
<td>GCTATTCAGGGCTTTTCTTC</td>
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<tr>
<td>actin revers primer (acR)</td>
<td></td>
<td></td>
<td>AGTACTTCAGGCAACGGAATC</td>
</tr>
<tr>
<td>FAE forward primer (FaF)</td>
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<td>59</td>
<td>TGCATGTAAGCGTAACGGAAC</td>
</tr>
<tr>
<td>CamV35S forward primer (CaF)</td>
<td></td>
<td></td>
<td>GCTCCTCAAAATCCATCA</td>
</tr>
<tr>
<td>NOS revers primer (NOR)</td>
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<td></td>
<td>GCAAGACCGCAACGGAATC</td>
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<td>virG forward primer</td>
<td>311</td>
<td>54-58</td>
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<tr>
<td>virG revers primer</td>
<td></td>
<td></td>
<td>CGCCAGCTTCGGCACATCAAG</td>
</tr>
</tbody>
</table>

ELISA Test for the Qualification and Quantification of Plant-based rHM

The ELISA test was performed following a procedure described previously.26 The sera against recombinant proteins were diluted to 1:200 in PBST and the standard curve was drawn using purified recombinant HM protein (from *Escherichia coli* BL21 (DE3)).26 All the samples were analyzed twice.

Parenteral and Oral immunization of Mice

We orally immunized 6 to 8 weeks old female BALB/c mice (Razi Vaccine and Serum Research Institute, Tehran, Iran) with the seeds of transgenic *B. napus* or hairy roots of *N. tabacum* separately. The mice were made to fast for 8 h prior to the oral immunization by gavage or ordinary feeding by the transgenic plants to mice. All immunized mice received transgenic proteins orally (equivalent to 10 μg antigenic recombinant protein/dry weight of *B. napus* seeds and 50 μL sodium bicarbonate or hairy roots of *N. tabacum* mixed with raw wheat flour and sugar) at weekly intervals for five weeks. Prime-boost mice were immunized similarly to oral groups; except the last gavage/feeding that was replaced with a subcutaneous injection of 10 μg purified bacterial rHM. The control mice were administered with a soluble protein extract made from non-transgenic *B. napus* seeds and *N. tabacum* hairy roots. Table 2 and Table 3 summarize the mice groups and a certain amount of administered transgenic plant biomass containing 10μg recombinant protein, respectively.

Antibody Response in Serum of Immunized Mice

Blood samples were collected from immunized mice one week after the last two immunizations by facial bleeding. The specific IgG and IgA antibodies against rHM, rHER2, and rMUC1 were analyzed by ELISA test as described in the previous study.26 All animal experiments were approved by the Animal Experimentation Committee of NIGEB (IR.NIGEB.EC.1396.7.25.B).

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Table 2. Groups of immunized mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Types of antigen expressed within transgenic plants</th>
<th>Administration route</th>
<th>Antigen dose (μg)</th>
<th>Immunization schedule (day)</th>
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</thead>
<tbody>
<tr>
<td>HM G</td>
<td>HM</td>
<td>Oral</td>
<td>10</td>
<td>0, 7, 14, 21, 28</td>
</tr>
<tr>
<td>HM P.B</td>
<td>HM</td>
<td>Prime-boost</td>
<td>10</td>
<td>0, 7, 14, 21, 28</td>
</tr>
<tr>
<td>HER2 G</td>
<td>Her2</td>
<td>Oral</td>
<td>10</td>
<td>0, 7, 14, 21, 28</td>
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<tr>
<td>HER2 P.B</td>
<td>Her2</td>
<td>Prime-boost</td>
<td>10</td>
<td>0, 7, 14, 21, 28</td>
</tr>
<tr>
<td>MUC1 G</td>
<td>MUC1</td>
<td>Oral</td>
<td>10</td>
<td>0, 7, 14, 21, 28</td>
</tr>
<tr>
<td>MUC1 P.B</td>
<td>MUC1</td>
<td>Prime-boost</td>
<td>10</td>
<td>0, 7, 14, 21, 28</td>
</tr>
<tr>
<td>Control</td>
<td>non-transgenic plant</td>
<td>Oral</td>
<td>non-transgenic</td>
<td>0, 7, 14, 21, 28</td>
</tr>
</tbody>
</table>

G: oral group. P.B: a prime-boost group that received Ag several times orally and last time by S.C injection. In all prime-boost groups, 10μg HM protein was injected subcutaneously as prime-boost. Five mice were used in each seed or root administrated group (a total of 70 mice).

Table 3. Amount of administered transgenic plant biomass containing 10 μg recombinant proteins no need to more explanation

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Transgenic hairy roots of N. tabacum (g)</th>
<th>Transgenic seeds of B. napus (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM</td>
<td>0.33</td>
<td>2.1</td>
</tr>
<tr>
<td>Her2</td>
<td>0.25</td>
<td>1.7</td>
</tr>
<tr>
<td>MUC1</td>
<td>0.38</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Cytokine and Lymphocyte Proliferation Assays

Four weeks after the last immunization, all mice from each group were euthanized and their spleen was dissected aseptically. The splenocytes were homogenized and suspended in an RPMI 1640 medium. IFNγ and IL-4 (Interleukin 4) were determined in the culture supernatants after 48 h of antigen stimulation using mouse ELISA kits by following the manufacturer’s instructions (Minneapolis, USA). All the assays were performed in triplicate. Lymphocyte proliferation was evaluated by MTT assay.27

Statistical Analysis

The data from protein assessment, antibody detection, and cytokine assay were analyzed using IBM SPSS Statistics software produced by SPSS Inc the one-way analysis of variance. p<0.01 was considered statistically significant.

RESULTS

Design and Cloning of the Synthetic HM Gene

The details of the gene design are presented in Figure 2. The elicited structure was analyzed using bioinformatics software as described previously.22 Since Her2 and MUC1 oncoproteins are considered as breast cancer antigens,3,5 we postulated that a bivalent antigen made from Her2-MUC1 antigenic domains in a unique chimeric recombinant protein (which could preserve the equal amount of two antigens in the same time) could stimulate the immune response more effectively than each of them alone.

Comparative expression studies of the multimeric protein complex HM in plant

A. tumefaciens LBA4404 harboring recombinant pBI1400(with fatty acyl elongase1 promoter as seed preferred promoter in Brassica napus28) or pBI121 plasmid (with Cauliflower mosaic virus promoter as constitutive promoter in different plant hosts,29) transgenic plants transformed with pBI1400-HM, pBI1400-Her2, pBI1400-MUC1, pBI121-HM, pBI121-Her2, and pBI121-MUC1; and non-transgenic plants were analyzed by PCR using the specific primers to detect the presence and integration of the recombinant genes. The authentic transgenes were selected for protein analysis using the quantitative ELISA test. The expression level was estimated to be approximately 0.015% of the total soluble protein (TSP) (Figure 3a) in all canola seeds transformed with HM, Her2, and MUC1 genes and was 0.57%, 0.45%, and 0.65% of TSP (Figure 3b) in the hairy roots of N. tabacum transformed with HM, Her2, and MUC1 genes, respectively. The transgenic seeds and hairy roots being used for the analysis of immunization in the animal model are shown in Table 3.
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Figure 3. Quantitative ELISA assay by Anti 8-His Tag for the measurement of recombinant HM, HER2, and MUC1 (containing 8-His Tag at the end of the amino acid sequence) in the transgenic plants (a. canola seeds and b. hairy root of *N. tabacum*). In all samples, the OD of wild type plants were deducted from that of the transgenic lines. Data are expressed as mean±SEM.

Figure 4. ELISA results for HM (a), HER2 (b), and MUC1 (c), specific IgG from the mice immunized orally by the recombinant proteins that were expressed in *B. napus* and HM (d), HER2(e) and MUC1 (f), specific IgG from mice immunized orally with recombinant proteins that were expressed in *N. tabacum*. The serum samples were collected from the mice after receiving 50μg the recombinant proteins. The sera were serially diluted and assayed using bacterial purified recombinant HM as the antigen. Data are expressed as mean ± SEM.
Figure 5. ELISA results for HM (a), HER2 (b) and MUC1 (c) specific IgA from the mice immunized by the recombinant proteins expressed in B. Napus and HM(d), HER2 (e) and MUC1 (f), the recombinant proteins expressed in N. tabacum. The serum samples were collected from the mice after receiving 50μg of recombinant proteins. Data are expressed as mean±SEM.

Figure 6. IFN-γ (a), IL-4 (b) levels and IFN-γ/IL4 ratio (c) in the cell supernatants were determined by ELISA. Different letters (A, B, C, D, E, F, G, H and I) represent significant differences between the groups (p<0.01). (P.B and G: the mice those received protein orally plus prime-boost dose and only orally, respectively; M: MUC1, H: HER2 and HM: HER2-MUC1 and C: control. Concanavalin A was used as a positive control and PBS as negative one). Data are expressed as mean±SEM.
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![Image](http://ijaai.tums.ac.ir)

Figure 7. Lymphocyte proliferation assay of splenocytes from the mice immunized with recombinant proteins. Different letters (A, B, C, D, E, F, G, H and I) represent a significant difference between groups (p<0.01). (P.B and G: the mice those received protein orally plus prime-boost dose and only orally, respectively; M: MUC1, H: HER2 and HM: HER2-MUC1 and C: control. Concanavalin A was used as a positive control and PBS as negative one). Data are expressed as mean±SEM.

Induction of Humoral Immune Response in Orally Immunized Mice

To evaluate the ability of transgenic B. napus seeds or N. tabacum hairy roots in raising humoral responses, the antisera of immunized mice were measured for specific IgG and IgA antibodies. The serum analysis showed that antibody titers in all immunized mice were evidently higher than that in the control group (p<0.01). This result shows that high IgG and IgA induction can be achieved using HM protein instead of using HER2 or MUC1 protein alone (Figure 4 and Figure 5). Based on statistical analysis no significant difference was observed in the antibody titer of the mice immunized with transgenic seeds from B. napus via gavage and those immunized with N. tabacum hairy roots by ordinary feeding (p>0.05).

Cytokine Assay

The supernatants of splenocyte cultures from the immunized and control mice were analyzed by ELISA for IFNγ and IL–4 cytokines as typical cytokines for Helper T lymphocyte 1 (Th1) and Helper T lymphocyte 2 (Th2) pattern. A considerable production of IFNγ and IL–4 was observed in the cells of immunized mice compared with that of control mice (p<0.01) (Figure 6a and b). IFNγ to IL–4 ratios showed that FNγ production was markedly higher than IL–4 in immunized mice (p<0.01)(Figure 6c).

Lymphocyte Proliferation Assay

The results of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] proliferation assay was calculated as the stimulation index (S.I., count per minute of induced to uninduced splenocytes). The S.I. in all mice immunized with HM, MUC1, and HER2 was considerably higher than the control mice group (p<0.01) (Figure 7).

DISCUSSION

The Her2 antigen has been utilized in several studies. Knutson and co-workers in 2016 developed a degenerated Her2 subdominant epitope-based vaccine that could be useful in approximately 85 % of all patients. Based on Kemerovo’s investigation, Nicotiana benthamiana plant could produce Herceptin efficiently. This protein could inhibit the Her2+ cell proliferation and finally lead to postponement of breast cancer growth. Despite these interesting results, we know that there are some limitations for use plant as a host for bioactive peptide production. Generally, three main challenges are the selection of antigen and plant expression host, consistency of dosage, and manufacturing of vaccines according to Good Manufacturing Practice (GMP) procedures. The MUC1 as a common cancer epitope has been used in the design of several cancer vaccines candidates. Additionally, Palitzsch and colleagues synthesized MUC1 glycopeptide vaccines and analyzed their structure-activity relationships in immunization. Based on this study, a monoclonal antibody specifically distinguishing between the normal and tumor epithelial cells in human was thus generated. Pinkhasov and her coworkers in 2011 immunized MUC1-tolerant mice (MUC1.Tg (with transiently expressed LTB-MUC1 from N. benthamiana leaves) by injecting LTB-MUC1 intraperitoneally and observed the production of anti-MUC1 serum antibodies indicating the break of tolerance. This finding indicates that a plant-derived human tumor-associated antigen is equivalent to the human antigen in the context of immune recognition.

In this study, we designed and synthesized or produced a bivalent and two monovalent recombinant proteins containing a truncated form of an exposed region of Her2 and MUC1 in plant-based systems.
improve the efficient expression and accumulation of designed antigens the rare codons were eliminated and the destabilizing elements and unwanted poly A signals which could affect the stability of mRNA were corrected. In fact, the first stages of N-glycosylation are highly conserved between plants and mammals, whereas it is significantly different in late N-glycosylation steps in the Golgi apparatus of plants which leads to complex N-glycan with immunogenic properties. For solving this problem, the sequences with the least glycosylation sites are selected, as there is no N-glycosylation site in MUC1 motif and only one N-glycosylation site in Her2 motif with low probability (under the threshold). O-glycosylation pattern is structurally different in plants and humans. To overcome this issue, there was an effort to select sequences with the least O-glycosylation sites in both antigens. Nevertheless, there are several O-glycosylation sites in our recombinant protein, especially in the MUC1 motif. This problem was dissolved by removing the specific signal peptide which leads the newly synthesized polypeptide into the Golgi apparatus using this strategy the O-glycosylation, will not or will rarely, occur. Because of hyper or hypo glycosylation of MUC1 in normal and cancer tissues respectively, the non-glycosylation form of MUC1 could mimic the natural structure of MUC1 in cancerous tissue.

The expression of novel recombinant proteins, HM, Her2, and MUC1 were evaluated in B. napus and N. tabacum. The results showed the considerable production of recombinant proteins in some transgenic plants lines. The expression levels of HM fusion protein, Her2, and MUC1 were approximately 0.01% of the TSP in transgenic B. napus seeds and N. tabacum hairy roots under the control of FAE1 and CaMV3Ss promoter respectively and the recombinant proteins can be effectively recognized by the mice sera against bacterial rHM (Figures 4 and 5). This can confirm the existence of recombinant proteins in B. napus seeds and N. tabacum hairy roots. This result is in agreement with the comparative expression studies on different plant species including wheat, pea, rice, and tomatoes though the expression level varied in different plant species. The first report of an edible plant-based vaccine was a surface antigen of Streptococcus expressed in tobacco at 0.02% TSP level of transgenic leaves in 1990. Two HIV expression genes were expressed successfully in tomatoes under CaMV35s promoter. The presence of the produced proteins was proved by molecular analyses in various tissues of the transgenic plant including the ripe tomatoes and also in the second generation of it. In another study, the fibroblast growth factor, expressed under the control of a CaMV3Ss promoter, accumulated up to 0.04% of the TSP in soybean, but by using glycycin seed promoter, the yield reached up to 2.3% of the TSP. Amani and his co-workers indicated that the regulatory seeds preferred FAE1 promoter which expressed a sufficient amount of antigen, needed for immunization.

We have found that the IgG and IgA concentrations are high in the immunized mice serum of both oral and prime-boost groups (Figure 4 and Figure 5). This indicates that the plant-derived HM, Her2, and MUC1 can effectively induce the immune response. For more than one decade, researchers have discovered that the edible vaccines are more stable, effective and practical than purified immunogens in the enzymatic and acidic environment of the gastrointestinal tract. The synthetic antigen rHM produced in plants can stimulate the immune response of the animal model at a dose of 10μg. In agreement with other studies, the route of vaccine administration affected the type of immune responses. In all mice groups received immunogens orally, production of IgA was significantly more than counterparts group which received immunogen orally plus prime-boost dose because the immunogen stimulates mucosal immune system well. Also, mice groups received immunogens orally plus prime-boost does, production of IgG was significantly more than counterparts group which received immunogen orally because the prime-boost dose stimulate s humoral immune system appropriately. Multicomponent vaccines which present more than one immunogen to the immune system could be considered as the second generation of edible vaccines. In this approach, numerous antigens can be integrated and presented to microfold cells (M cells) in gut tissue simultaneously, and can stimulate the immune system more efficiently than injectable vaccines. As was expected, the increase in antibody titer was higher in the mice immunized with transgenic plants harboring the HM than in those immunized with the only MUC1, whereas it was more in the mice immunized with only MUC1 - producing transgenic plants than in those immunized with Her2. These findings show that the combination of two proteins stimulates the immune system more effectively than each protein separately. Furthermore,
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because of compromising repeated sequences in MUC1, this molecule could effectively stimulate the immune system. The MHC (Major Histocompatibility Complex)-unrestricted recognition of MUC1 by T cells can be explained by the fact that MUC1 has multiple repeated epitopes that can cross-link the TCR on the surface of the T cells.\(^{31}\) For developing the most effective T helper type–1 (Th1) immunity, naive CD4+ T cells must acquire the capacity to express IFN\(\gamma\) while controlling the potential of T helper type–2 (Th2) for cytokine-production.\(^{32}\) IFN\(\gamma\) is essential in maintaining the Th1 phenotype by actively silencing the IL4 gene transcription potential.\(^{33}\) Generally, the vaccination in breast cancer patients should stimulate CD8+ cytotoxic T lymphocytes (CTL) to remove tumor cells via recognition of surface tumor-associated antigenic (TAA) epitopes of cancer cells by TCRs. Also, optimal immunogen should stimulate CD4+helper T cells for regulating immune responses and cytotoxic responses triggering.\(^{34}\)

In our study, IFN\(\gamma\) in all test mice groups was considerably higher than control mice (Figure 6a). In agreement with the antibody analyses and our in silico predictions, IFN\(\gamma\) production in mice treated with HM was significantly more than MUC1, and in mice treated with MUC1 was more than Her2. Smorlesi and colleagues reported that the increased production of IFN\(\gamma\) by CD8+ T lymphocytes, the reduced production of IL–4 by CD4+ T cells, and the change in antibody phenotype observed in transgenic Her2 positive mice vaccinated with DNA vaccine against Her2, clearly indicated the enhancement of Th1 type immune responses.\(^{35}\) Catherine J. Lees and colleagues reported that immunotherapy with the oxidized mannan-MUC1 fusion protein (M-FP) leads to the generation of cytotoxic T lymphocytes (CTL), few antibodies, secretion of IFN\(\gamma\) and finally tumor protection. Immunotherapy with reduced M-FP or fusion protein alone leads to the generation of MUC1 antibodies and scarce CTL, IL–4 secretion, and no tumor protection.\(^{36}\)

In the current study, IFN\(\gamma\) to IL4 ratio showed that these recombinant proteins in all immunized groups considerably induced Th1 more than Th2 as compared with control groups (Figure 6c). The results of MTT assays confirmed our previous results stating that the HM immunogen was the strongest one, and MUC1 cause more efficient proliferation than Her2. Lymphocyte proliferation in immunized mice spleens was markedly higher than in the control group. Thus, it may be one of the reasons behind a strong immune response (Figure 7).

In sum, we can conclude that these transgenic seeds and hairy roots with the ability to produce Her2, MUC1, and HM tumor antigens can properly stimulate the humoral and cellular immune system in immunized mice vs control animals. Further studies are required to produce this novel chimeric protein in other raw edible plants such as tomato, lettuce, banana and kiwi for evaluating the efficacy of it in preventing many malignancies.

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