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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 as an injective vaccines in cancer therapy researches, but not in the chimeric form as an 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 IFNɣ cytokine. However, the animals received chimeric protein showed significant higher immune responses in comparison to ones received one of these immunogen.

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 vaccine; 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 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 the breast cancer.[3] The human mucin-like molecule, polymorphic epithelial mucin (MUC1), a tumor-associated antigen, is a polymorphic transmembrane glycoprotein expressed on theapical 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.[5] These qualitative and quantitative changes of MUC1 expression in cancer render it immunogenicity. Some studies have identified anti-MUC1 immune responses in preclinical investigations and cancer patients after activeimmunization with MUC1.[6,7]

For the production of transgenic Her2 and MUC1 polypeptides, selecting a suitable host is important. Among different heterologous hosts for the 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, 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 degradation in the upper digestive tract before they reach the gut-associated lymphoid tissue (GALT).[11] It is prove that the hairy roots can be used for producing recombinant proteins with the biosafety consideration such as the lack of pollination and control of gene transfer.[12] Another excellent plant tissue, 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 itshigh protein content (20% w/w) can be manipulated to produce high levels of recombinant heterologous proteins which is very suitable formore efficient delivery of an oral immunogen.[16] In the previous studies, the polypeptides from different parts of breast cancer antigens have been produced successfully in plant systems, injected into animal models and stimulated the immune responses,[17-19] However, it seems that chimeric protein which consists of more than one important breast cancer antigens could be more effective to defeat the disease. According to our literature review, there have been no studies performed on oraldelivery ofHer2 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 of production to more than eighty percent.[20,21]

In this regard, and based on our previous in silico analysis, 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) from MUC1 antigene, were analysed by bioinformatics softwares.[22] The multimeric gene was synthesized by ShineGene (China).

Construction of Plant Expression Vectors

The *hm* gene was subcloned in *Cfr*9I/*Sac*I sites of pBI1400[22] and in *Xba*I/*Sac*I sites of the pBI121 vector (Novagen, USA). In both vectors, the *β-glucuronidase* gene was replaced with the synthetic *hm* gene. The resulting plasmids (pBI1400-*hm* and pBI121-*hm*) were utilized for the transformation of *Brassica napus (B.napus)* and *Nicotianatabacum (N.tabacum)*, respectively. The recombinant authentic plasmids were separately introduced into *Agrobacterium tumefaciens* LBA4404cells.[23] The recombinant *A. Tumefaciens*cells were verified by PCR analysis and used for plant transformation. The separate genes encoding HER2 and MUC1 amplified from the original plasmid by standard PCR using Pfu DNA polymerase. The sequencing and digestion were performed to evaluate the integrity of these four (pBI1400 or pBI121 harboring HER2 or MUC1) constructs.

Plant Transformation and Regeneration

Commercial canola (*B.napus* L.cultivar PF 7045-91) and *N.tabacum* (cultivar Samsun) seeds obtained from the Seed and Plant Improvement Research Institute of Iran. The transformation of both plants was

carried outusing 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.[24] The hairy roots appeared after ten days and were cultured in liquid MS medium without any additive.[25]

PCR Analysis of Transgenic Plants

The presence of transgenes in putative transgenic plants were determined by PCR analysis on genomic DNA using specific primers (Table 1and Figure 1). The primers attachment and cutting sites of restriction enzymes shown in Figure 2.

ELISA Test for Qualification and Quantification of Plant-based rHM

The ELISA test was performed following a procedure described previously.[26] The sera against recombinant proteins werediluted 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

Eight weeks old female BALB/c mice (Razi Vaccine and Serum Research Institute, Tehran, Iran) were immunized with the seeds of transgenic *B.napus*or hairy roots of *N. tabacum*plants separately. The mice were fast for 8 h prior to the oral immunization by gavage or ordinary feeding by the transgenic plants. 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 administered with a soluble protein extract made from non-transgenic *B. Napus* seeds and *N. tabacum*hairyroots.

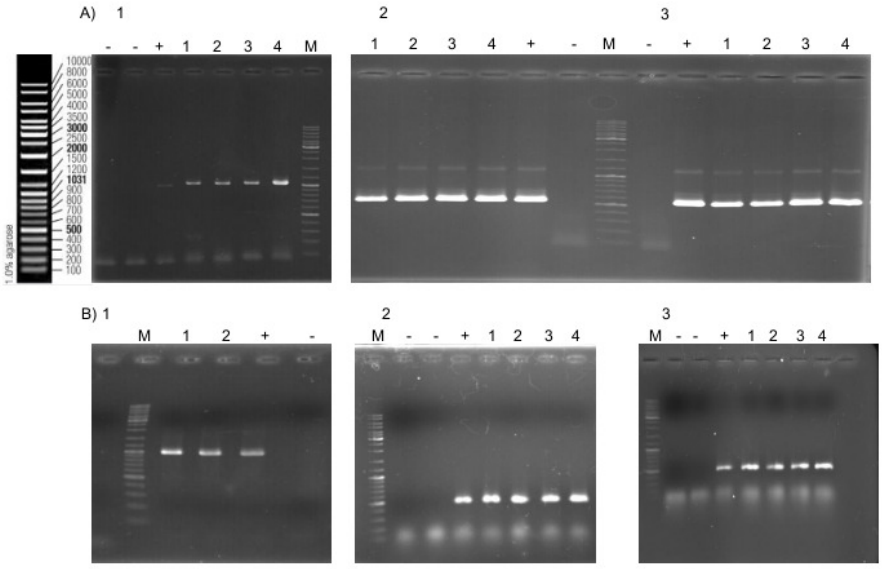

Figure 1. Molecular analysis of transgenic plants. A) Amplification of *HM* (A1, 1051bp) and *Her2* genes (A2, 653bp) in transgenic *B. napus* with the specific FAE (Fatty Acid Elongase) /NOS primers and *MUC1* gene (A3, 518bp) in transgenic *B. napus* with the specific *MUC1* primers. B) Amplification of *HM* (B1, 1017bp) with the specific *Her2/MUC1* primers and *Her2* genes (B2,477bp) specific *Her2* primers 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).

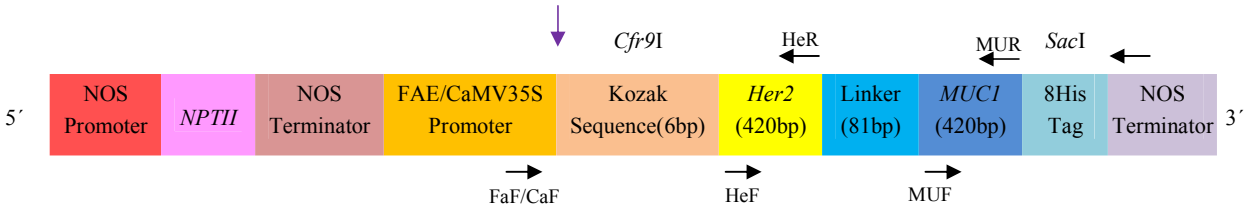

Figure2. Schematic representation of pBI1400-HM/pBI121-*hm*constructs. The constructs contain the *hm*gene consisting of coding sequences for the immuno-protective epitopes of the HER2 and MUC1 antigens under the control of the promoter [either FAE or CaMV35S (Cauliflower Mosaic Virus 35S)]. The primers' attachment sites are shown with arrows.

Table 1. The primers, sequences and products sizes.

primers' names	products sizes(bp)	Tm(^{0}C)	primers' sequences
Her2 forward primer (HeF)	465	60	AGATCTCCCGGG*TCTAGAAAAACAATGGCTCCTTGGG
Her2 revers primer (HeR)			TCAGCGAGCTC*TCAGAGTTCGTCTTTTTCCTCGTCTGGAAACTTCC
MUC1 forward primer (MUF)	486	56	AGGCACCCGGG*TCTAGAAAAACAATGGCTCCTGATACAAGGCCTG
MUC1 revers primer (MUR)			CAAATGTTTGAACGATCGGGGAAATTCGAGCTCTCAGAGTTCGTC
actin forward primer (acF)	380	57	GCTATTCAGGCCGTTCTTTCTC
actin revers primer (acR)			AGTACTTCAGGGCAACGGAATC
FAE forward primer (FaF)	It depends on the size of the cloned fragment	59	TGCATGTAAAGCGTAACGGACC
CamV35S forward primer (CaF)			GCTCCTACAAATGCCATCA
NOS revers primer (NOR)			GCAAGACCGGCAACAGGATTC
virG forward primer	311	54-58	TAGGTCGTGAAGATGGGCTC
virG revers primer			CGCCAGCTTCGGACATCAAG

* The underlined show the restriction enzyme sites.

Table 2 and Table 3 summarize the mice groups and amount of transgenic plant biomass containing 10 µg recombinant protein, respectively.

Antibody Response in Serum of Immunized Mice

Blood sample 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 previously.[26] All animal experiments were approved by the Animal Experimentation Committee of NIGEB (IR.NIGEB.EC.1396.7.25.B).

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 RPMI1640 medium.

IFNγ and IL–4 (Interleukin 4) were determined in theculture supernatants after 48 hof antigen stimulation using mouse ELISA kits by following the manufacturer's instructions (Minneapolis, USA). All the assays were performed intriplicate. 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 analysisof variance. $p<0.01$ was considered statistically significant.

RESULTS

Design and Cloning of the Synthetic *hm* Gene

The details of the gene design presented in Figure 2. The synthetic structure was analyzed using bioinformatics software as described previously.[22]

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 napus)*[28] or pBI121 plasmid (with Cauliflower mosaic virus promoter as constitutive promoter in different plant hosts;)[29] were used for plant transformation. the transgenic plants harboring pBI1400-*HM*, pBI1400-*Her2*, pBI1400-*MUC1*, pBI121-*HM*, pBI121-*Her2*, and pBI121-*MUC1*plasmids; and non-transgenicplants were analyzed by PCR using the specific primers to detect the presence and integration of the recombinant genes. The authentic transgenes 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 amount of transgenic seeds and hairy roots used for immunization study are shown in Table 3.

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

Table 2. Groups of immunized mice

Groups	Types of antigen expressed in transgenic plants	Administration route	Antigen dose	Immunization schedule (day)
HM G	HM	Oral	10 µg	0, 7, 14, 21, 28
HM P.B	HM	Prime-boost	10 µg	0, 7, 14, 21, 28
HER2 G	Her2	Oral	10 µg	0, 7, 14, 21, 28
HER2 P.B	Her2	Prime-boost	10 µg	0, 7, 14, 21, 28
MUC1 G	MUC1	Oral	10 µg	0, 7, 14, 21, 28
MUC1 P.B	MUC1	Prime-boost	10 µg	0, 7, 14, 21, 28
Control	non-transgenic plant	Oral	non-transgenic plant	0, 7, 14, 21, 28

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

Table 3. Amount of administered transgenic plant biomass containing 10 µg recombinant proteins

Antigens	Transgenic hairy roots of *N. tabacum* (g)	Transgenic seeds of *B. napus* (g)
HM	0.33	2.1
Her2	0.25	1.7
MUC1	0.38	1.9

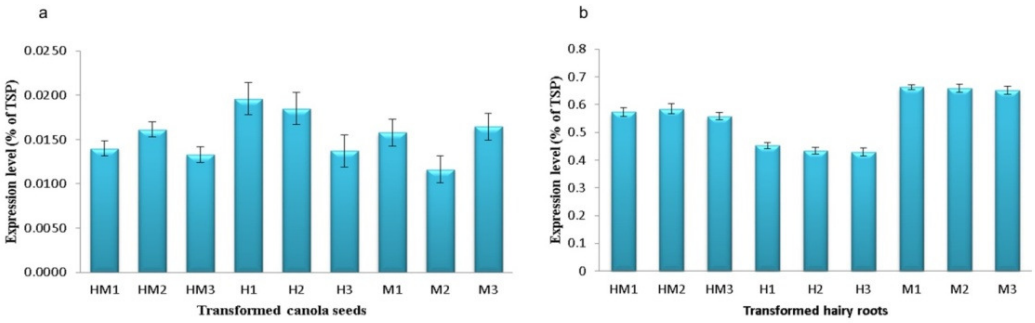

Figure3. Quantitative ELISA assay by Anti His Tag for the measurement of recombinant HM, HER2, and MUC1 in the transgenic plants (a. canola seeds and b. hairy root of *N.tabacum*). In all samples, the OD of wild type plants were deducted from that of the transgenic lines. Data expressed as mean±SEM.

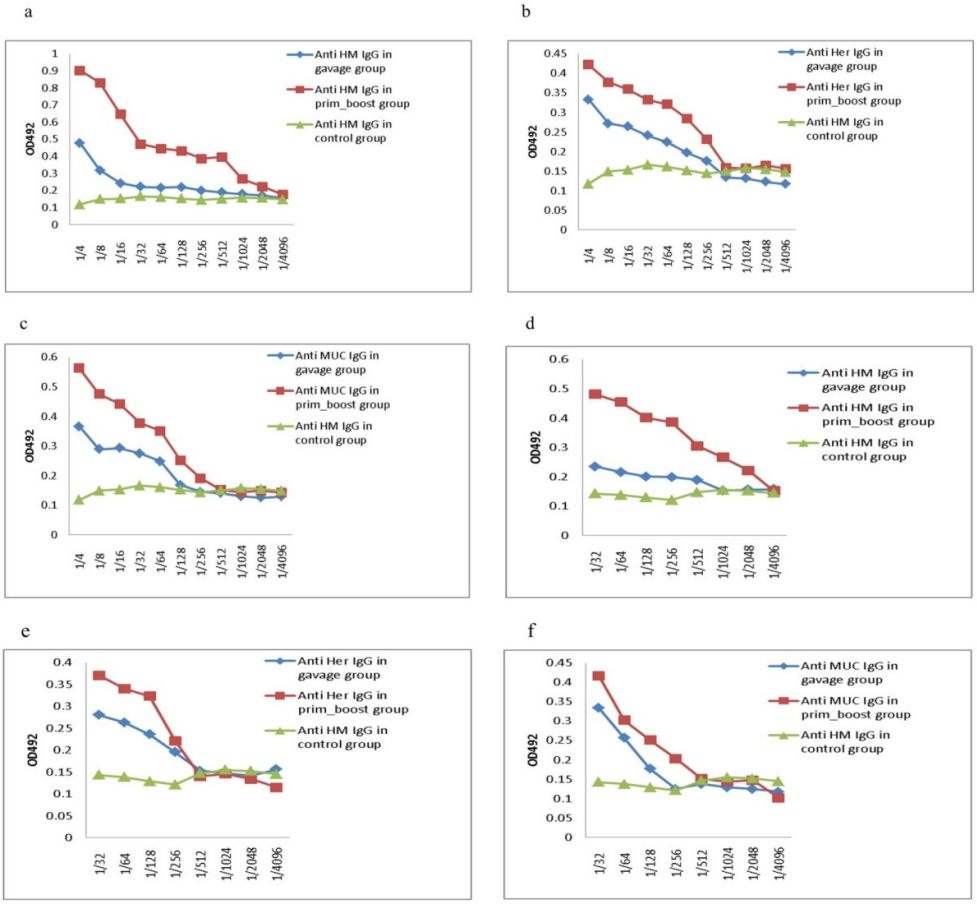

Figure4. ELISA results for HM (a), HER2 (b), and MUC1 (c), specific IgG from the mice immunized orally by the recombinant proteins were expressed in *B.Napus* seedsand HM (d), HER2 (e) and MUC1 (f), specific IgG from mice immunized orally with recombinant proteins were expressed in *N.tabacumhairy roots*. The mice sera were serially diluted and assayed using bacterial purified recombinant HM protein as the antigen. Data were expressed as mean ± SEM.

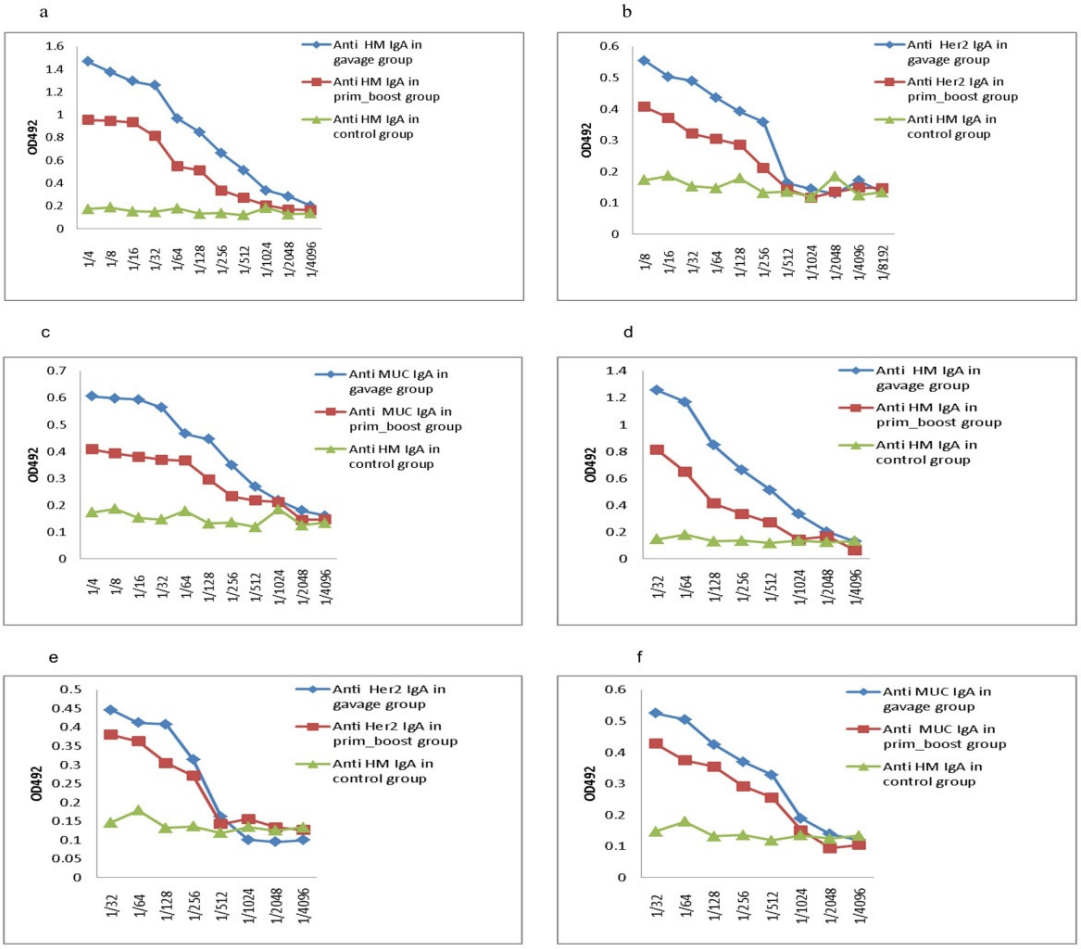

Figure5. ELISA results for HM (a), HER2 (b) and MUC1 (c) specific IgA from the mice immunized with recombinant proteins expressed in *B.Napusseeds* and HM (d), HER2 (e) and MUC1 (f), the recombinant proteins expressed in *N.tabacum*. Data were expressed as mean±SEM.

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 splenocytes 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 group (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 uninducedsplenocytes). The S.I. in all mice immunized with HM, MUC1, and HER2 was considerably higher thanthe control mice group (p<0.01) (Figure 7).

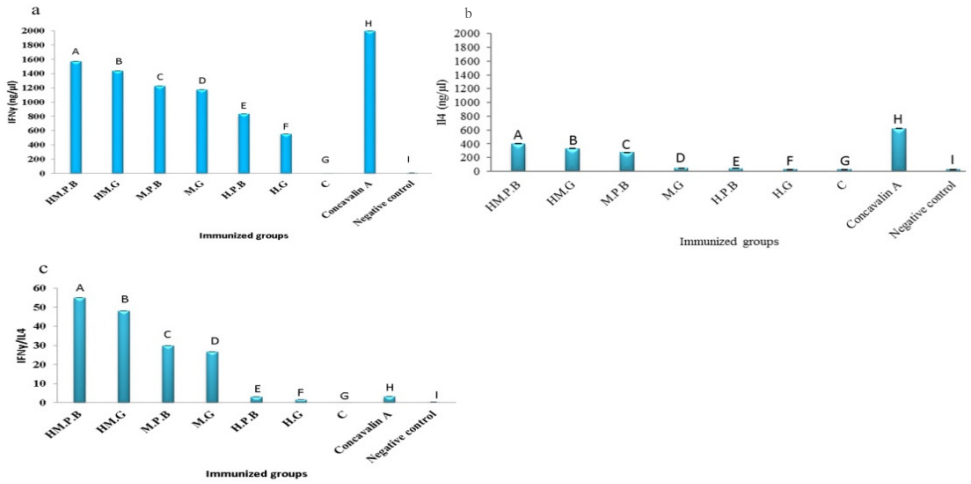

Figure6. IFN-γ (a), IL–4 (b) levels and IFN-γ/IL4 ratio (c) in the cell supernatants were determined by ELISA. Different letters (A, B, C, D, E, F, G, H and I) represent significant differences between the groups (p<0.01). (P.B and G: the mice those received protein orally plus prime-boostdose and only orally, respectively; M: MUC1, H: HER2 and HM: HER2-MUC1 and C: control. Concanavalin A was used as a positive control and the PBS as negative one). Data expressed as mean±SEM.

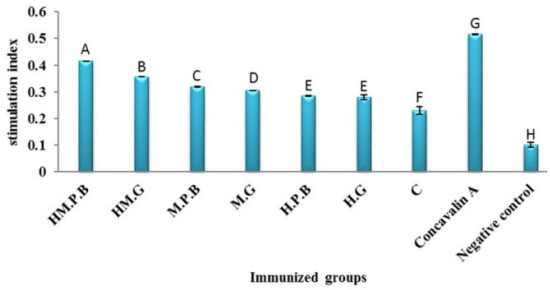

Figure7. Lymphocyte proliferation assay of splenocytes from the mice immunized with recombinant proteins. Different letters (A, B, C, D, E, F, G, H and I) represent a significant difference between groups (p<0.01). (P.B and G: the mice those received protein orally plus prime-boostdose and only orally, 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 expressed as mean±SEM.

DISCUSSION

The Her2 an important antigen in breast cancer has been utilized in several studies.[30,31] Knutson and co-workers in 2016 developed a degenerated Her2 subdominant epitope-based vaccine that could be useful in approximately 85 %of allpatients.[32] Based on Kemerovo's investigation, *Nicotiana benthamiana* plant could produce mono clonal antibody against Her2 antigene, Herceptin, efficiently. This specific antibody could inhibit the Her2+cell proliferation and finally lead to postponement of breast cancer growth.[33] Despite these interesting results, we know that there are some limitations for use plant as a host for bioactive peptide production. The selection of appropriate antigen and plant expression host, consistency of dosage, and manufacturing of vaccines according to Good Manufacturing Practice (GMP) procedures are the main challenges in transgenic plant production system.[34] The MUC1 as a common cancer epitope has been used in the design of several cancer vaccines candidates.[35,36] 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.[37] 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 in traperitoneally and reported 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 thehuman antigen in the context of immune recognition.[38]

Here, we designed , synthesized and produced a

bivalent and two monovalent recombinant proteins containing a truncated form of exposed region of Her2 and MUC1 in plant-basedsystems.[11] To improve the efficient expression and accumulation of designed antigens the rare codons were eliminated and the destabilizing elements and unwanted cis elements such as poly A signals which could affect the stability of mRNA were corrected.[11] We know that the first stages of N-glycosylation are highly conserved between plants and mammals. But it is significantly different in late N-glycosylation steps in the Golgi apparatus of plants which leads to complex N-glycan with immunogenic properties.[39,40] 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.[40] 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. Due to 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 evaluated in *B. napus* and *N.tabacum*. The results showed the considerable amount 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 CaMV35s 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 presence 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, peas, rice, and tomatoes though the expression level varied in different plant species.[41-44] 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.[45] Two HIV expression genes were expressed successfully in tomatoes under the control of CaMV35s promoter. The presence of heterologous proteins was proved by molecular analyses in various tissues of the transgenic plant including the ripe tomatoes. The stability of the recombinant antigene has been shown in the second generation.[46] In another study, the fibroblast growth factor, expressed under the control of a CaMV35S promoter, accumulated up to 0.04% of the TSP in soybean, but replacing the CaMV35S with glycinin seed promoter, increased the yield up to 2.3% of the TSP.[44] Amani and his co-workers indicated that the regulatory seeds preferred FAE1 promoter could express sufficient amount of recombinant antigen which is needed for animal model immunization.[47,48] The ELISA test shows in figure 3 the level of recombinant HM, HER2 or MUC1in transgenic seeds are rather low (0.015 TSP), but due to high protein content of canola seeds, this amout is sufficient for immunization procedure.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 oral delivery or edible vaccine could protect the antigene structure which is crucial for conformational epitope formation. Further more the antigens are more stable and effective than purified immunogens in the enzymatic and acidic environment of the gastrointestinal tract.[49] The synthetic antigen rHM produced in plants can stimulate the immune response of the animal model at a low dose of 10 µg. In agreement with other studies, the route of vaccine administration affected the type of immune responses.[27] In all mice groups received immunogens via oral rout, the production of IgA was significantly more than other group which received immunogen orally plus one injection as a prime-boost dose. Also, mice groups received immunogens orally plus prime-boost does, significantly could produce more IgG than counterparts group which received immunogen orally. It seems the prime-boost dose could stimulates humoral immune system appropriately. Multicomponent vaccines which present more than one immunogen to the immune system could be considered as the newgeneration of edible vaccines. In this approach, numerous antigens

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can be integrated and presented to microfold cells (Mcells) in gut tissue simultaneously,[50] and can stimulate the mucosal immune system more efficiently than injectable vaccines.[46] 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 MUC1 and this one was more effective than HER2. These findings show that the combination of two proteins stimulates the immune system more effectively than each protein separately. Further more, the presence of repeatedmotif in MUC1, able this molecule to stimulate the immune system, effectively. We know that the MUC1 has multiple repeated epitopes which could cross-link the TCR on the surface of the T cells and produce more effective immune responces.[51] For developing the most effective T helper type–1 (Th1) immunity, naive CD4+ T cells must acquire the capacity to express IFNγ while controlling the potential of T helper type–2 (Th2) for cytokine-production.[52] IFNγ is essential in maintaining the Th1 phenotype by actively silencingthe IL4 gene transcription potential.[53] 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. The optimal immunogen should stimulate CD4+helper T cells for regulating immune responses and cytotoxic responses triggering.[54]

In our study, IFNγ in all test mice groups was considerably higher than control mice (Figure 6a). In agreement with the antibody analyses and our in silico predictions, IFNγ production in mice treated with HM was significantly more than animal group receive MUC1 and this group produce more cytokine than mice group treated with HER2. Smorlesi and colleagues reported that the increased production of IFNγ 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 Th1type immune responses.[55] 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 IFNγ and finally tumor protection. Immunotherapy with reduced M-FP or fusion protein alone leads to the generation of MUC1 antibodies and scarce CTL, IL–4secretion, and finally no protection against tumor.[56] In the current study, IFNγ 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 the MUC1 molecule cause more efficient proliferation than Her2. Lymphocyte proliferation in immunized mice spleens was markedlyhigher than in the control group (Figure 7).

Finally 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 animal models. Further studies are required to evaluate the efficiency of this immunity for protection or breast cancer treatment. Further more, production of this novel chimeric protein in other raw edible plants and fruits such as tomato, lettuce and banana is necessary.

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