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The rat ErbB2 tyrosine kinase receptor produced in plants is immunogenic in mice and confers protective immunity against ErbB2$^+$ mammary cancer

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Summary

The rat ErbB2 (rErbB2) protein is a 185-kDa glycoprotein belonging to the epidermal growth factor-related proteins (ErbB) of receptor tyrosine kinases. Overexpression and mutations of ErbB proteins lead to several malignancies including breast, lung, pancreatic, bladder and ovary carcinomas. ErbB2 is immunogenic and is an ideal candidate for cancer immunotherapy. We investigated the possibility of expressing the extracellular (EC) domain of rErbB2 (653 amino acids, aa) in *Nicotiana benthamiana* plants, testing the influence of the 23 aa transmembrane (TM) sequence on protein accumulation. Synthetic variants of the *rErbB2* gene portion encoding the EC domain, optimized with a human codon usage and either linked to the full TM domain (rErbB2_TM, 676 aa), to a portion of it (rErbB2-pTM, 662 aa), or deprived of it (rErbB2_noTM, 653 aa) were cloned in the pEAQ-HT expression vector as 6X His tag fusions. All rErbB2 variants (72–74.5 kDa) were transiently expressed, but the TM was detrimental for rErbB2 EC accumulation. rERbB2_noTM was the most expressed protein; it was solubilized and purified with Nickel affinity resin. When crude soluble extracts expressing rErbB2_noTM were administered to BALB/c mice, specific rErbB2 immune responses were triggered. A potent antitumour activity was induced when vaccinated mice were challenged with syngeneic transplantable ErbB2$^+$ mammary carcinoma cells. To our knowledge, this is the first report of expression of rErbB2 in plants and of its efficacy in inducing a protective antitumour immune response, opening interesting perspectives for further immunological testing.

Introduction

Cancer is a leading cause of death worldwide, and a major unmet need is to radically improve the survival of cancer patients and find new therapies. Nowadays, surgery, radiation and chemotherapy are the main anticancer weapons, often causing adverse side effects. Cancer immunotherapy harnesses the body's immune system to fight against diverse tumours, without developing resistance (Couzin-Frankel, 2013), but can be successful only if it targets an oncoantigen, a tumour-associated antigen with strong oncogenic properties and unlike to escape immune recognition (Iezzi *et al.*, 2012; Lollini *et al.*, 2006). ErbB2 is a 185-kDa transmembrane protein with tyrosine kinase activity, that is expressed at low levels in normal tissue but overexpressed in 25%–30% of breast cancers and in other malignancies (Kern *et al.*, 1990; Lei *et al.*, 1995; Sato *et al.*, 1992; Slamon *et al.*, 1989). ErbB2 is the main oncoantigen for breast cancer (Lollini *et al.*, 2006). Current treatment of ErbB2$^+$ positive cancers includes passive administration of monoclonal antibodies (Mab) against ErbB2, use of drugs that inhibit its enzymatic activity (den Hollander *et al.*, 2013), or the recently approved antibody–drug conjugates (Peddi and Hurvitz, 2014). ErbB2 is immunogenic (Napoletano *et al.*, 2009; Quaglino *et al.*, 2004, 2011) and vaccines inducing antibodies against it are under study (Baselga *et al.*,1998; Jacob *et al.*, 2006; Li *et al.*, 2013; Quaglino *et al.*, 2010).

Plants are regarded as biotechnological tools for producing candidate vaccines against human or animal diseases (Chen, 2008; Rybicky, 2010); plant-made antigens have been already tested in clinical trials and administered orally, with successful outcomes (Kapusta *et al.*, 1999; Pniewski *et al.*, 2011; Tacket *et al.*, 1998). Plant cells can produce single or multiple proteins that can assemble in high-order structures also serving as protein expression platforms to present heterologous epitopes (Matić *et al.*, 2011; Thuenemann *et al.*, 2013). In this work, we investigated the expression in *Nicotiana benthamiana* plants of the extracellular (EC) moiety of the rat ErbB2 (rErbB2) oncoantigen and tested the immunogenicity and antitumour activity of the plant-made protein.

Results

The EC domain of rErbB2 can be transiently expressed in *N. benthamiana*

To express the *rErbB2* gene in plants, we started with the sequence encoding the EC domain to avoid the risks of

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manipulating the full protein and particularly the oncogenic intracellular region. Moreover, considering the membrane anchoring role of the transmembrane (TM) domain (23 aa) in mammalian cells, three gene variants were tested for expression in plants, that is *rErbB2_TM* (encoding the EC and TM domain, aa 1-676), *rErbB2_pTM* (encoding the EC and only 9 aa of the TM domain, aa 1-662) and r*ErbB2_noTM* (encoding only the EC moiety, aa 1-653). All variants were synthetically produced *in vitro* with optimized human codons. Synthetic genes were cloned in the pEAQ-HT vector, downstream of a 6X His-tag coding sequence (Figure 1). Following agro-infiltration, leaf extracts were analysed in Western blot using Mabs specific for ErbB2 and the His tag. After direct extraction in Laemmli sample buffer, polypeptides of about 72–73 kDa were detected only in tissue infiltrated with the rErbB2_pTM and rErbB2_noTM constructs (Figure 2a). To ascertain whether the lack of rErbB2_TM detection derived from lower expression or an intrinsic difficulty of extraction, leaf tissue samples were treated by a trichloroacetic acid (TCA)/acetone procedure. By this, the amount of extractable rErbB2_pTM and rErbB2_noTM proteins increased, and traces of rErbB2_TM became detectable (Figure 2a). Using known amounts of a recombinant rErbB2 protein from *E. coli* as a quantification standards, yields of 5, 60 and 280 μg per mg of fresh leaf tissue were calculated for rErbB2_TM, rErbB2_pTM and rErbB2_noTM, respectively.

The yield of rErbB2_noTM increased until approximately 6 days post infiltration (dpi) (Figure 2b) and was maintained for about 10 days, with occasional yellowing and necrosis of the infiltrated area (not shown).

The above results indicate that the EC moiety of rErbB2 can be successfully expressed in plants, but the TM domain negatively influences its accumulation. Based on yield, all further assays were carried out with rErbB2_noTM.

rErbB2_noTM solubilization and purification

To prepare plant extracts containing rErbB2_noTM in a soluble form suitable for mice immunizations, leaf tissue expressing the recombinant protein was incubated with either phosphate or Tris–HCl buffers at neutral pH, containing different combinations of ionic or nonionic detergents (i.e. Tween-20, Triton X-100 or SDS at 0.05%–0.5%). Many plant proteins were soluble in these conditions, but rErbB2_noTM was never detected in soluble fractions (not shown), unless 5 mM DTT and either 300 mM NaCl or 8 M urea were added to the extraction mixtures (see Experimental procedure, Buffers A and B and Figure 3a).

The His-tagged rErbB2_noTM (72.5 kDa) was purified from crude soluble extracts made in a buffer containing 300 mM NaCl using Nickel affinity resin (Figure 3b). The additional high molecular weight molecules that eluted from the column and reacted with the anti-ErbB2 Mab (Figure 3b) may be aggregates

(a)

```
            1                                                                                                  100
rErbB2_noTM MELAAWCRWGFLLALLPPGIAGTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYVPANASLSFLQDIQEVQGYMLIAHNQVKRVPLQRLR
rErbB2_pTM  MELAAWCRWGFLLALLPPGIAGTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYVPANASLSFLQDIQEVQGYMLIAHNQVKRVPLQRLR
rErbB2_TM   MELAAWCRWGFLLALLPPGIAGTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYVPANASLSFLQDIQEVQGYMLIAHNQVKRVPLQRLR
            101                                                                                                200
rErbB2_noTM IVRGTQLFEDKYALAVLDNRDPQDNVAASTPGRTPEGLRELQLRSLTEILKGGVLIRGNPQLCYQDMVLWKDVFRKNNQLAPVDIDTNRSRACPPCAPAC
rErbB2_pTM  IVRGTQLFEDKYALAVLDNRDPQDNVAASTPGRTPEGLRELQLRSLTEILKGGVLIRGNPQLCYQDMVLWKDVFRKNNQLAPVDIDTNRSRACPPCAPAC
rErbB2TM   IVRGT QLFEDKYALAVLDNRDPQDNVAASTPGRTPEGLRELQLRSLTEILKGGVLIRGNPQLCYQDMVLWKDVFRKNNQLAPVDIDTNRSRACPPCAPAC
            201                                                                                                300
rErbB2_noTM KDNHCWGESPEDCQILTGTICTSGCARCKGRLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMHNPEGRYTFGASCVTTC
rErbB2_pTM  KDNHCWGESPEDCQILTGTICTSGCARCKGRLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMHNPEGRYTFGASCVTTC
rErbB2_TM   KDNHCWGESPEDCQILTGTICTSGCARCKGRLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMHNPEGRYTFGASCVTTC
            301                                                                                                400
rErbB2_noTM PYNYLSTEVGSCTLVCPPNNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLRGARAITSDNVQEFDGCKKIFGSLAFLPESFDGDPSSGIAPLRPEQLQV
rErbB2_pTM  PYNYLSTEVGSCTLVCPPNNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLRGARAITSDNVQEFDGCKKIFGSLAFLPESFDGDPSSGIAPLRPEQLQV
rErbB2_TM   PYNYLSTEVGSCTLVCPPNNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLRGARAITSDNVQEFDGCKKIFGSLAFLPESFDGDPSSGIAPLRPEQLQV
            401                                                                                                500
rErbB2_noTM FETLEEITGYLYISAWPDSLRDLSVFQNLRIIRGRILHDGAYSLTLQGLGIHSLGLRSLRELGSGLALIHRNAHLCFVHTVPWDQLFRNPHQALLHSGNR
rErbB2_pTM  FETLEEITGYLYISAWPDSLRDLSVFQNLRIIRGRILHDGAYSLTLQGLGIHSLGLRSLRELGSGLALIHRNAHLCFVHTVPWDQLFRNPHQALLHSGNR
rErbB2_TM   FETLEEITGYLYISAWPDSLRDLSVFQNLRIIRGRILHDGAYSLTLQGLGIHSLGLRSLRELGSGLALIHRNAHLCFVHTVPWDQLFRNPHQALLHSGNR
            501                                                                                                600
rErbB2_noTM PEEDCGLEGLVCNSLCAHGHCWGPGPTQCVNCSHFLRGQECVEECRVWKGLPREYVSDKRCLPCHPECQPQNSSETCFGSEADQCAACAHYKDSSSCVAR
rErbB2_pTM  PEEDCGLEGLVCNSLCAHGHCWGPGPTQCVNCSHFLRGQECVEECRVWKGLPREYVSDKRCLPCHPECQPQNSSETCFGSEADQCAACAHYKDSSSCVAR
rErbB2_TM   PEEDCGLEGLVCNSLCAHGHCWGPGPTQCVNCSHFLRGQECVEECRVWKGLPREYVSDKRCLPCHPECQPQNSSETCFGSEADQCAACAHYKDSSSCVAR
            601                                                653       662           676
rErbB2_noTM CPSGVKPDLSYMPIWKYPDEEGICQPCPINCTHSCVDLDERGCPAEQRASPVT-----------------------
rErbB2_pTM  CPSGVKPDLSYMPIWKYPDEEGICQPCPINCTHSCVDLDERGCPAEQRASPVTFIIATVVGV--------------
rErbB2_TM   CPSGVKPDLSYMPIWKYPDEEGICQPCPINCTHSCVDLDERGCPAEQRASPVTFIIATVVGVLLFLILVVVVGILI
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(b)

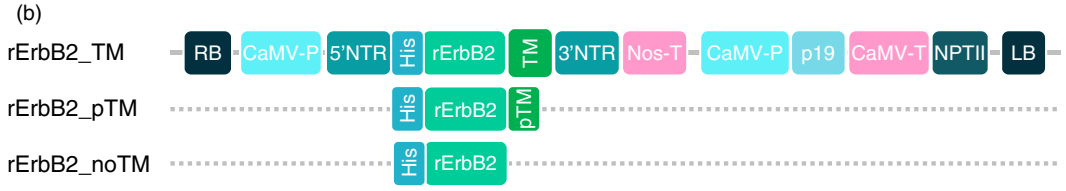

Figure 1 rErbB2 variants and constructs description. (a) ClustalW alignment of the EC domains of the rErbB2 proteins, with the TM domain indicated in green. (b) Scheme of the T-DNA regions within the right and left borders (RB and LB). The *rErbB2* synthetic genes fused to the 6X His tag (His) of pEAQ-HT are under the control of the CaMV promoter (CaMV-P), between the 5′ and 3′ untranslated regions (NTR) of the RNA-2 of *Cowpea mosaic virus* and the nopaline synthase terminator (Nos-T). pEAQ-HT also includes the silencing suppressor p19, under CaMV promoter and terminator (CamMV-P and -T).

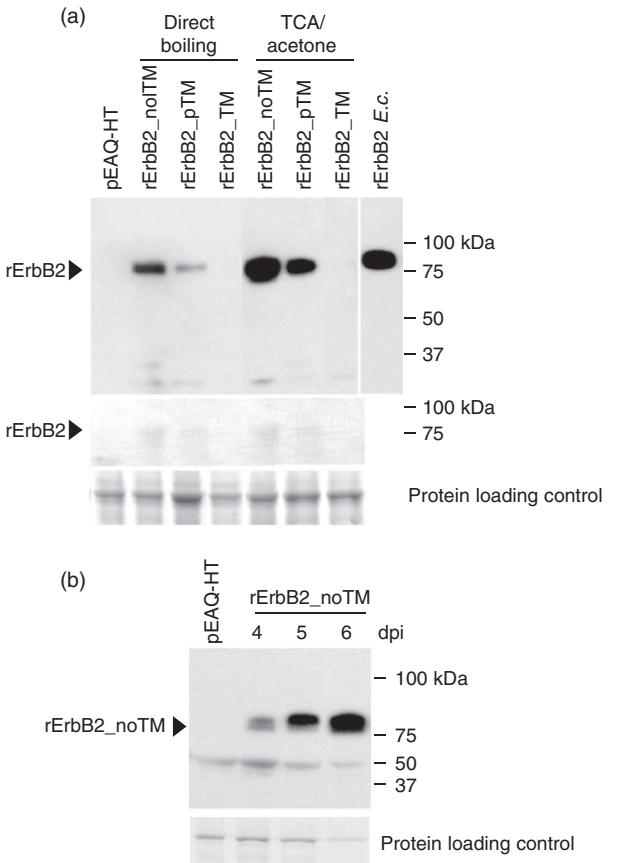

Figure 2 Transient expression in *N. benthamiana* of the synthetic *rErbB2* genes fused to 6X His tag. (a) Comparative Western blot of total proteins extracted from leaf samples (0.5 mg fresh tissue/lane) infiltrated with the three *rErbB2* variants or with the empty pEAQ-HT (control) at 5 dpi, probed with the anti-ErbB2 or the anti 6X His Mabs (upper and middle panel, respectively). The large subunit of Rubisco is shown as loading control (lower panel). rErbB2 is indicated with an arrowhead. An aliquot of 330 ng of rErbB2 protein produced in *E. coli* (Cat. No# 028823286; Genway) was loaded as quantification standard. (b) Time course of rErbB2_noTM accumulation (4–6 dpi). In both panels, protein molecular weight standards are indicated on the right (in kDa).

derived from misfolded ErbB2 polypeptides or, alternatively, ErbB2 oligomers; it is known that ErbB2 forms biologically active dimers/oligomers when overexpressed in mammalian cells and the EC domain alone is sufficient for such multimerization (Brennan *et al.*, 2000). For the low yield of purified protein obtained (about 2 μg/g fresh leaf tissue), crude soluble extracts prepared in buffer A were used for mice immunizations; buffer A was preferred for the absence of chaotropic agents.

Soluble plant-derived rErbB2_no TM elicits specific anti-rErbB2 antibodies

To determine the immunogenicity of the plant-made rErbB2_noTM, soluble extracts containing the recombinant protein were administered to BALB/c mice by subcutaneous injection (12 μg of plant-derived rErbB2 per dose). Animals vaccinated with control extracts infiltrated with the empty pEAQ-HT plasmid did not develop any specific response; instead, a significant amount of anti-rErbB2 antibodies was detected in sera of mice vaccinated with rErbB2_noTM (Figure 4a and b). In spite of the evident and measurable humoral immune response elicited in mice by the plant rErbB2 protein, only a negligible cytotoxic-specific response against cells pulsed with the immune-dominant rErbB2 peptide (TYVPANASL) was determined in all mice (Figure S1) by an *in vivo* cytotoxicity assay (Quaglino *et al.*, 2010).

Mice immunized with plant-derived rErbB2_noTM exhibit antitumour activity

The antitumour activity of the plant-made rErbB2_noTM protein was evaluated by *in vivo* tumour rejection experiments, challenging vaccinated mice with a lethal dose of TUBO cells. A growing tumour occurred in all mice vaccinated with control extracts (empty pEAQ-HT plasmid; $n = 9$), whereas a tumour developed only in one of eight mice vaccinated with extracts expressing rErbB2_noTM (Figure 4c). Moreover, the time required by this single tumour to exceed a 10-mm threshold was 55 days, while the same tumour size was reached after about 33 ± 2 days in mice treated with the empty pEAQ-HT vector (Figure 4d). Importantly, mice vaccinated with extracts expressing the rErbB2_noTM protein necroscopied at the end of the experiment were free from overt signs of autoimmune lesions in the heart, kidney and liver (data not shown).

Discussion

The potential low production costs, combined to minor biological risks, ease of scaling up and the ability of plants to provide an endomembranous system suitable for protein folding, assembly and post-translational modifications similar to mammalian systems are interesting advantages of plant expression platforms (Ma *et al.*, 2005; Thuenemann *et al.*, 2013). No current recombinant expression strategies offer the cheapness and speed of protein production, such as transient expression in plants. Transient expression is based on the delivery of the T-DNA region carrying the gene(s) of interest from *Agrobacterium tumefaciens* to the plant nucleus, followed by transcriptional and translational activation without T-DNA integration into the plant nuclear genome. Agrobacteria carrying the gene(s) of interest can be delivered into the intercellular spaces of parenchyma through agroinfiltration (Gelvin, 2003; Kapila *et al.*, 1997), leading in few days or weeks to the accumulation of proteins *in planta* at yields suitable for industrial-scale production (Marillonnet *et al.*, 2005; Sainsbury and Lomonossoff, 2008). Here, we investigated how to properly express an active portion of the rErbB2 receptor in plants, using a transient expression system based on a simple and efficient vector (Sainsbury *et al.*, 2009). We clearly demonstrated that following agroinfiltration of *N. benthamiana* with vectors carrying synthetic sequences encoding the EC domain of a humanized *rErbB2* gene, significant yields of rErbB2 protein are achieved.

Many factors contribute to the accumulation of a recombinant protein in plants, among which its structure, stability and solubility that ultimately depend also on its route of destination into the cell and interference with host cell components (Barbante *et al.*, 2008; Streatfield, 2007).

The TM sequence that in our constructs was present at the C-terminus of the EC domain of rErbB2 was expected to provide membrane anchorage of the protein and possibly have a stabilizing effect. However, such TM domain was detrimental for rErbB2 accumulation. The structure of the three variants predicted using the RaptorX server (http://raptorx.uchicago.edu/) was not significantly altered when the TM was partially or totally deleted (not shown). An extraction procedure more suitable for

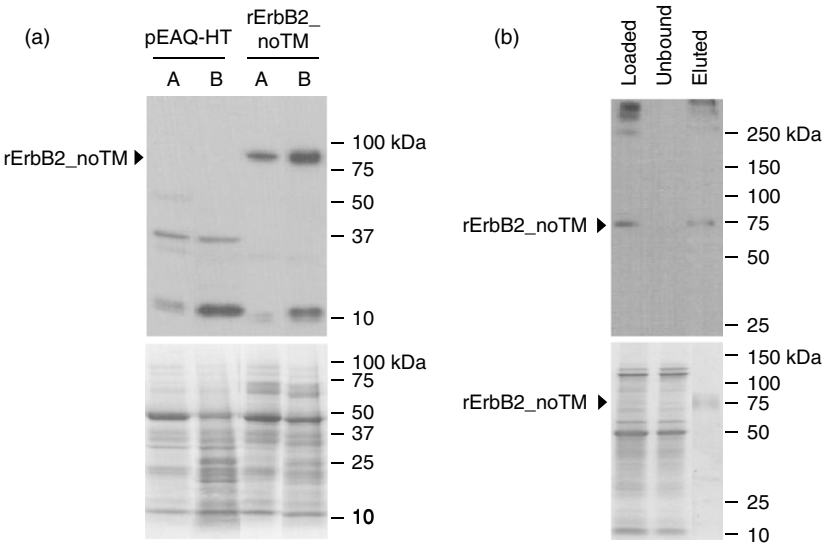

Figure 3 Preparation of soluble extracts for mice immunization and protein purification. (a) Protein solubilization. Leaf tissue agroinfiltrated with pEAQ-HT or rErbB2_noTM was extracted in buffers A and B (see Experimental procedure); soluble fractions were denatured in Laemmli sample buffer and tested in Western blot using the anti-ErbB2 antibody; a Coomassie stained gel is shown below as quality and loading control. (b) Purification of rErbB2_noTM by Nickel affinity beads. Aliquots from the different purification steps were denatured and tested in Western blot; a Coomassie stained gel is shown below. In both panels, protein molecular weight standards are indicated on the right (in kDa).

membrane-bound proteins (Wu and Wang, 1984) improved the pulling out of rErbB2 polypeptides from the plant tissue, allowing rErbB2_TM detection, though only in traces. This was suggestive of a different localization of rErbB2_noTM and rErbB2_TM within the plant cell. *In silico* subcellular targeting analyses of the EC rErbB2 sequences with or without the TM domain predicted similar destinations for the two proteins, that is mitochondrion or secretory pathways using the TargetP 1.1 server (www.cbs.dtu.dk/services/TargetP) or plasma membrane with the LocTree server (https://rostlab.org/services/loctree2). Conversely, the PSort tool (http://psort.hgc.jp/) was discriminatory, predicting the mitochondrial inner membrane, chloroplast thylakoid, or plasma membrane for rErbB2_TM (scores 0.9, 0.7 and 0.7, respectively) and the mitochondrial matrix space for rErbB2_noTM (score 0.7). Our attempts to localize and discriminate the rErbB2 variants in plant cells suffered from a poor level of expression of the green fluorescent protein (GFP)-rErbB2 fusions compared to GFP alone (Supplementary Materials in Appendix S1 and Figure S2a) that justified the addition of a silencing suppressor protein during agroinfiltration. Laser scanning confocal fluorescence analysis of tissue expressing GFP alone or the GFP-rErbB2_noTM and GFP-rErbB2_TM fusion proteins did not reveal clear differences between the two recombinant proteins that could prove a differential cellular targeting (Figure S2b), leaving still open the discussion on the negative effect of the TM sequence on rErbB2 yield.

The efficient expression system employed, coupled to a simple solubilization procedure, allowed us to obtain appropriate amounts of rErbB2_noTM in <2 weeks. Recently, the expression of the EC domain of human ErbB2 in *Pichia pastoris* and its biological activity were reported (Dimitriadis *et al.*, 2009). Notably, the yeast-made protein migrated as a 120- to 210-kDa polypeptide, interpreted as indicative of heavy mannosylation. As the EC domain of ErbB2 expressed by transduced human cells (that cannot perform mannosylation) has no antineoplastic role, while the yeast-derived protein had significant antitumour effect in mice, the authors concluded that mannosylation was an essential trait (Dimitriadis *et al.*, 2009). An *in silico* analysis of glycosylation in eukaryotes with the GlycoEP server (Cauhan *et al.*, 2013) predicted six potentially *N*-glycosylated residues for the rErbB2 EC domain (i.e. N68, N188, N260, N531, N572 and N630), the first five of which were also predicted by the NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc). Although the plant-made rErbB2_noTM still awaits a biochemical characterization, its migration as a 72-kDa polypeptide let us to speculate that no heavy glycosylation had occurred. The potent protecting effect of such protein in mice following challenge albeit with rErbB2$^+$ tumour cells allows to reasonably conclude that its strong immunogenicity and protecting activity occur irrespective of its glycosylation pattern.

As anti-rErbB2 antibody production was the prominent immune response induced by vaccination, we speculate that the plant-made rErbB2 EC moiety exposes native conformational epitopes in a correct configuration. The vaccine formulation used in this study elicited a significant and effective humoral antitumour immune response, albeit with negligible cellular cytotoxicity (Figure S1A). This failure could be explained considering the nature of the vaccine; as a soluble protein, peptides derived from it are presented to T cells mainly through MHC class II antigen, thus triggering CD4$^+$, but not CD8$^+$ T cells. Of course, cross-presentation can occur, but probably in a way insufficient to induce a measurable cytotoxic T-cell response. Constructs encoding fusion proteins consisting of the ErbB2-EC domain linked to a molecular moiety that facilitates antigen uptake and presentation by dendritic cells could be designed to elicit significant T-cell response (Sloots *et al.*, 2008; Zizzari *et al.*, 2011).

This work represents one of the first successful assays to use plant-expressed vaccines against cancer, providing a reagent suitable to design further immunotherapeutic strategies for anti tumour vaccination purposes. Conclusively, our data could be of impact for the scientific community, speeding up the translation of a new, nontoxic management modality to treat human cancer patients, hopefully contributing to improve their survival.

Experimental procedures

Gene synthesis and cloning of rErbB2

Three genes encoding the EC domain of the rErbB2 protein linked to a full (TM) domain (1965 nt) (*rErbB2_TM*), a partial TM (1923 nt) (*rErbB2_pTM*) or without TM (1896 nt) (*rErbB2_noTM*) (Figure 1a) were synthesized with an optimized human codon usage by GeneArt (Thermo Fisher Scientific, Waltham, MA) (*rErbB2_TM*) or GenScript (Piscataway, NJ) (*rErbB2_pTM* and *rErbB2_noTM*). Genes were subcloned into the plant expression

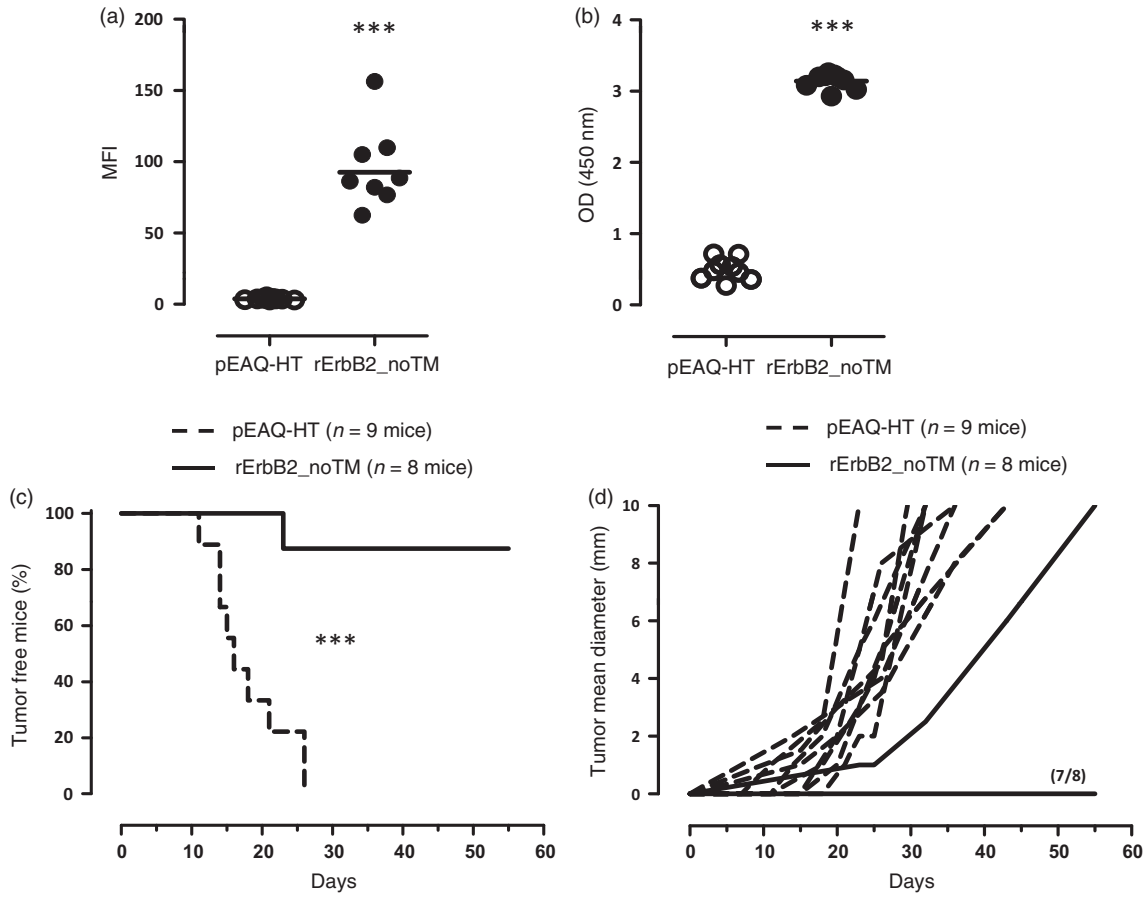

Figure 4 Antibody and antitumour response against rErbB2 induced by plant-derived rErbB2_noTM immunization. (a, b) Induction of specific anti-rErbB2 antibodies in sera of BALB/c mice immunized with extracts from leaves infiltrated with pEAQ-HT (n = 9) or the rErbB2_noTM (n = 8) plasmid measured by flow cytometry (a) and ELISA (b). Anti-rErbB2 antibody titres are expressed as mean fluorescence intensity (MFI) or optical density (OD) values for each vaccinated mice. Horizontal lines represent the geometric mean of each experimental group. ***P < 0.0001 (Student's t-test). (c) Antitumour protection against TUBO cells. Tumour incidence of BALB/c mice vaccinated with extracts from leaves infiltrated with the recombinant pEAQ-HT (n = 9; dotted lines) or rErbB2_noTM (n = 8; continuous lines) plasmid. ***P = 0.0001 (log-rank Mantel–Cox test). (d) Tumour speed of growth. Each line refers to an individual tumour; the number of mice that rejected the tumour/total mice in the group of rErbB2_noTM vaccinated mice is in brackets.

vector pEAQ-HT (Sainsbury *et al.*, 2009) (Figure 1b) fused to the sequence encoding the 6XHis tag.

Transient expression in plants

Constructs made in pEAQ-HT were transformed into *A. tumefaciens* LBA4404 and delivered to *N. benthamiana* plants by agroinfiltration (Noris *et al.*, 2011), using the empty pEAQ-HT vector as control. Plants were grown at 23 °C (16/8 h light/dark). At the appropriate times, agroinfiltrated tissue was homogenized in Laemmli sample buffer (9 μL/mg fresh leaf) and boiled for 2 min. Alternatively, total proteins were extracted from infiltrated tissue using trichloroacetic acid (TCA, extensively washed with acetone (Wu and Wang, 1984) and denatured in Laemmli sample buffer. Protein electrophoresis and Western blot were performed as described (Matić *et al.*, 2011), using anti-ErbB2 Mab (WH0002064M6; Sigma, St. Louis, MO, 1 : 1000 dilution) or anti-6XHis Mab (Thermo Fisher Scientific, Rockford, IL, 1 : 2000 dilution), in parallel experiments. The plant-derived rErbB2 antigen was quantified by comparing the intensity of the signal detected in Western blot with that of a series of dilutions of a recombinant rErbB2 protein produced in *E. coli* (cat#1028823286; Genway, San Diego, CA), ranging from 33 to 660 ng/lane, used as standard. Quantification of signal intensities was performed with the ImageJ software (http://imagej.nih.gov/ij/).

Preparation of soluble protein extracts and rErbB2_noTM purification

Leaves expressing rErbB2_noTM were collected 5 dpi, lyophilized, homogenized with liquid N_2 and extracted (33 μL/mg dry tissue) with Buffer A (50 mM Na-phosphate buffer, pH 8.0, 300 mM NaCl, 10 mM EDTA, 2.5 mM DTT, 0.5 mM PMSF, 0.2% Tween-20) or Buffer B (8 M urea, 10 mM EDTA, 2.5 mM DTT, 0.5 mM PMSF in PBS). After a brief incubation, homogenates were centrifuged (15300 g, 15 min, 4 °C) and supernatants stored at −20 °C. For protein purification, leaf tissue was extracted in a modified Buffer A including 10 mM imidazole, but lacking DTT and EDTA, loaded in batch onto Ni-NTA Agarose beads (Qiagen Inc., Valencia, CA). Proteins were eluted with 100 mM imidazole, following manufacturer instructions.

Mice immunizations

BALB/c female mice (Charles River, Calco, Italy) were bred at the Molecular Biotechnology Center (Torino, Italy) and treated according to the European Guidelines and policies, as approved by the University of Torino Ethical Committee. Two groups of

10-week-old mice received three subcutaneous injections at biweekly intervals with 150 μL leaf extracts expressing rErbB2_noTM or infiltrated with pEAQ-HT (control). Extracts were emulsified with equal volumes of complete or incomplete Freund's adjuvant (Sigma), immediately before injection. Mice were bled 2 weeks after the third vaccination, and sera were stored at −20 °C.

Anti-rErbB2 antibody immune response

Sera from immunized mice were tested by flow cytometry for their ability to bind NIH3T3 murine fibroblasts expressing rErbB2 (3T3/NKB cells; 25) and by ELISA. For flow cytometry, 3T3/NKB cells were cultured in DMEM with Glutamax 1 (Life Technologies, Monza, Italy) supplemented with 20% heat-inactivated fetal bovine serum (FBS), G418 and zeocin (all Invitrogen, Monza, Italy; the last two 0.6 mg/mL). 3T3/NKB cells were pretreated with Fc receptor blocker (CD16/CD32; BD Biosciences, Milano, Italy) (15 min, 4 °C) and incubated (30 min, 4 °C) with 20-fold diluted mice sera. Total IgG binding was evaluated using a FITC-conjugated goat anti-mouse IgG Fc antibody (DakoCytomation, Glostrup, Denmark). The Ab4 Mab (Oncogene Research Products, La Jolla, CA) was used as positive control. Flow cytometry was performed with a CyAn ADP (DakoCytomation, Beckman Coulter, Milano, Italy). Results are expressed as mean fluorescence intensity (MFI) and analysed with Summit 4.2 (DakoCytomation) software.

For ELISA, 96-well plates (Costar®; Sigma-Aldrich) were coated with 100 ng per well of recombinant rErbB2 protein from *E. coli* (Genway) overnight at 4 °C. Coated plates were then blocked with 10% Newborn Calf Serum (NCBS; Sigma-Aldrich) in PBS (Invitrogen)-0.05% Tween (Sigma-Aldrich) buffer for 2 h at 37 °C. Plates were incubated with mice sera diluted 20-fold in 1% blocking buffer for 1 h at 37 °C. Plates were washed three times with a PBS-Tween buffer. The HRP-conjugated anti-mouse IgG antibody (Sigma-Aldrich) (1 : 2000 dilution in blocking buffer) was incubated for 1 h at 37 °C. Plates were washed six times, and chromogenic 3,3′,5,5′-tetramethylbenzidine substrate was added (TMB; Sigma-Aldrich). The reaction was stopped by the addition of 2N HCl; optical density (OD) was measured at 450 nm using a microplate reader (680XR; Bio-Rad, Richmond, California, USA). Differences in MFI and OD were analysed by Student's *t*-test.

Anti tumour protection against rErbB2$^+$ mammary TUBO cells

Two weeks after the third immunization, mice were challenged subcutaneously (s.c.) with a lethal dose (1×10^5) of rErbB2$^+$ mammary carcinoma cells derived from a mammary carcinoma arisen in a BALB/c female mouse transgenic for the activated *rErbB2* gene (BALB-neuT mice; Quaglino *et al.*, 2008). TUBO cells were cultured in DMEM with Glutamax 1 (Life Technologies) plus 20% heat-inactivated FBS (Invitrogen). Mice were weekly inspected by palpation for tumour growth, regarding progressively growing masses >1 mm in diameter as tumours. Mice were sacrificed when one of the tumours exceeded 10 mm diameter. Differences in tumour incidence were analysed by the log-rank (Mantel–Cox) test.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Specific anti-rErbB2 immune response induced by plant-derived rErbB2_noTM immunization.

Figure S2 Transient expression and localization in plant cells of green fluorescent protein (GFP) and GFP-rErbB2 fusion variants.

Appendix S1 Supplementary Materials and Methods.

