Timely DNA Vaccine Combined with Systemic IL-12 Prevents Parotid Carcinomas before a Dominant-Negative p53 Makes Their Growth Independent of *HER-2/neu* Expression[1]

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Double transgenic mice overexpressing the transforming rat *HER-2/neu* oncogene and the mutated p53, with both dominant-negative and a gain-of-function properties, display early aggressive and metastasizing parotid tumors. Multiple acinar and ductal hyperplasia foci overexpressing the *HER-2/neu* gene product are evident at wk 5 and progress to poorly differentiated carcinoma by wk 7. Mice die before wk 18 with invasive carcinomas and multiple metastases that no longer express HER-2/neu. A combination of repeated electroporations of plasmids coding for the extracellular and transmembrane domains of the rat HER-2/neu receptor with systemic IL-12 administrations started when the parotids that present diffuse hyperplasia protected all female and 50% of the male mice until the close of the experiment at wk 40. This combined treatment began when multifocal in situ carcinomas that were already present cured 33% of the females and 25% of the males. The most prominent immunologic features associated with the antitumor protection were the production of high titers of anti-HER-2/neu Abs and the nonappearance of cell-mediated cytotoxic reactivity. In conclusion, anti-HER-2/neu vaccination combined with systemic IL-12 control parotid carcinomas as far as *p53* mutation makes their growth independent of HER-2/neu expression. ***The Journal of Immunology,*** **2006, 176: 7695–7703.**

While the overall incidence of salivary carcinomas is 2.5–3.0/100,000 persons/year in Western countries (1–3), the possibility to impair their expansion or prevent their onset through a specific immunity has been hardly ever evaluated. Yet these carcinomas frequently express an amplification of the *ErbB-2* (*HER-2*) oncogene, and the membrane overexpression of its protein product (p185)[4] is common and correlates with a poor prognosis (4–7). p185 is a receptor tyrosine kinase related to the epidermal growth factor receptor family. Its overexpression leads to the formation of homo- and heterodimers that deliver mitogenic signals to the cells and are thus responsible for the initiation and progression of neoplastic transformation (8–11).

BALB-neuT transgenic mice carrying the transforming rat (r-) *HER-2* oncogene under the transcriptional control of the mouse mammary tumor virus overexpress the r-p185 in their mammary, salivary, and harderian glands and the epididymis. However, all female BALB-neuT mice develop lethal mammary carcinomas (12), whereas indolent acinar parotid adenocarcinomas arise sporadically in aging males (13).

Inactivation of *p53* is another common genetic event of human salivary and breast carcinomas (14–17). As *p53* deficiency synergizes to promote *HER-2* carcinogenesis (17), we first generated BALB-neuT mice with knocked out *p53* gene (BALB-neuTp53 KO). However, as these mice develop simultaneously more than one type of tumor, the potential of a vaccine to cure and prevent salivary carcinomas cannot be neatly teased apart (18).

In the present study, BALB-neuT mice were made transgenic for a mutated form of *p53* (BALB-neuT/p53$^{172R\text{-}H}$ mice) that more closely resembles the human Li-Fraumeni syndrome (19). Substitution of aa 172 from arginine to histidine (R-H) is the mouse equivalent of the most common p53 alteration (175, R-H) in humans. It results in a gain-of-function and not just a dominant-negative phenotype (20). Transgenic female mice expressing p53$^{172R\text{-}H}$ targeted by a whey acidic protein promoter showed no apparent alterations in normal salivary and mammary development. Even so, they displayed increased genomic instability and developed mammary carcinomas with shorter latency and faster kinetics than their control littermates in response to dimethylbenz(*a*)anthracene (21). The *p53*$^{172R\text{-}H}$ mutant allele predisposes mice to a spectrum of tumors that is distinct from that produced by null alleles (22). Moreover, *p53* null mice develop less aggressive tumors (23).

Male and female BALB/c mice combining *r-HER-2* and *p53*$^{172R\text{-}H}$ overexpression (BALB-neuT/p53$^{172R\text{-}H}$ mice) quickly develop fast-growing and highly metastasizing parotid gland carcinomas, whereas the slow progression of their mammary tumors is similar to that of parental BALB-neuT mice and they do not develop mesenchymal tumors. While the cells of early stages of parotid gland neoplastic growth, namely ductal and acinar hyperplasia, strongly overexpress membrane and cytoplasmic r-p185, in situ

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Received for publication June 11, 2005. Accepted for publication March 31, 2006.

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[1] This work was supported by grants from Italian Association for Cancer Research, the Italian Ministries for the Universities and Health, the University of Torino, Compagnia di San Paolo, Torino, and the Center of Excellence on Aging, University of Chieti, Italy.

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[4] Abbreviations used in this paper: p185, HER-2 transgene protein; r-, rat; MSA, mouse serum albumin; EC-TM, transmembrane and extracellular domains of r-p185; Spc, spleen cell; DC, dendritic cell; PCNA, proliferative cell nuclear Ag.

0022-1767/06/$02.00

carcinomas are less intensely r-p185$^+$, and invasive, poorly differentiated carcinomas almost completely lose r-p185 expression. BALB-neuT/p53^{172R-H} thus provide a realistic model for determination of the preventive and curative efficacy against an aggressive salivary carcinoma of a simple and well-defined vaccination regimen that has proved effective against slow growing and constantly r-p185$^+$ mammary tumors (24). In these mice, systemic administration of IL-12 combined with i.m. DNA electroporation of plasmids coding for the extracellular and transmembrane domains of the r-p185 (EC-TM plasmids) persistently prevented carcinoma onset in most mice. The most prominent immunologic features were the production of high titers of anti-r-p185 Abs and the nonappearance of cell-mediated cytotoxic reactivity. The reduced ability to inhibit the growth of multifocal in situ carcinomas goes along with their progressive reduced expression of r-p185.

Materials and Methods

BALB-neuT/p53^{172R-H} mice

BALB-p53^{172R-H} were obtained by crossing BALB/c wild-type mice (Charles River Laboratories) with FVB/NTgN(Trp53R172H)8512Jmr mice (The Jackson Laboratory) for more than 12 generations. BALB-neuT transgenic mice carrying the activated *r-HER-2* oncogene under the transcriptional control of the mouse mammary tumor virus enhancing/promoter have been generated in our laboratories (12). BALB-neuT/p53^{172R-H} mice generated by crossing BALB-p53^{172R-H} females with BALB-neuT males are hemizygous for both the activated *r-HER-2* transgene and the murine p53^{172R-H} transgene driven by the whey acidic protein promoter. They were assigned randomly to the control and treatment groups. Similar results were obtained when each experiment was repeated two to three times. The data were cumulated in the figures and tables. Mammary and parotid glands were inspected weekly to note tumor appearance. Tumor masses were measured with calipers in two perpendicular diameters. Progressively growing masses $>$ 1 mm in mean diameter were regarded as tumors. Mice were treated according to the European Union guidelines and to protocols approved by the University of Torino Review Board.

IL-12 administration

Recombinant mouse IL-12 (provided by Dr. S. Wolf, Genetics Institute, Cambridge, MA) was diluted in PBS supplemented with 0.01% mouse serum albumin (MSA; Sigma-Aldrich) and administered i.p. as described previously (12, 25). Starting from the fifth or seventh week of age, mice received a course of one weekly i.p. injection of 0.2 ml of PBS containing MSA plus 100 ng of IL-12, for 4 wk, followed by a 3-wk rest. This course was repeated throughout their life.

Preparation of EC-TM plasmids and in vivo electroporation

The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen Life Technologies) by deleting the SV40 promoter, neomycin resistance gene, and SV40 poly(A). The sequence for the the extracellular (EC) and transmembrane (TM) domains of the protein product of mutated r-p185 were generated from the PCR product using the primers 39-CGCAAGCTTCAT CATGGAGCTGGC-59 and 39-CGGAATTCGGGCTGGCTCTCTGC TC-59 and the primers 39-CGCAAGCTTCATGGAGCTGGC-59 and 39-ATGAATTCTTTCCGCATCGTGTACTTCTTCCGG-59, respectively, as previously described (26). PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hin*dIII and *Eco*RI, and cloned into the multiple cloning site of the pCMV plasmid to obtain the EC-TM plasmid used in this work. The leader sequence is the same as r-p185 (63 bp from the ATG starting codon of the cloned cDNA). DNA was precipitated, suspended in sterile saline at 1 mg/ml, and stored in aliquots at 20°C for use in immunization protocols (27, 28). Twenty-five micrograms of empty and EC-TM plasmids in 20 μl of 0.9% NaCl with 6 mg/ml polyglutamate were injected bilaterally into the tibial muscle of the hind legs of anesthetized mice. Two 25-ms transcutaneous electric pulses were generated by a T820 electroporator (BTX) with a field strength of 375 V/cm, as previously described in detail (24, 29). Plasmids were electroporated during the first and third week of each IL-12 course and at the same times in the mice that received plasmid electroporation only.

Morphological analyses

For histologic evaluation, tissue samples were fixed in 10% neutral-buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E. For immunohistochemistry, paraffin-embedded or acetone-fixed cryostat sections were immunostained with anti-p185 (clone C-18) (Santa Cruz Biotechnology), anti-proliferative cell nuclear Ag (PCNA) (clone PC10) (DakoCytomation), anti-tenascin C (clone MTn-12) (Abcam), and anti-endothelial cells (CD31, PECAM-1, clone MEC13.3) (BD Pharmingen), Abs. After washing, sections were overlaid with biotinylated goat anti-rat and anti-rabbit Ig (Vector Laboratories) for 30 min. Unbound Ig was removed by washing, and slides were incubated with avidin-biotin complex /alkaline phosphatase (DakoCytomation). For double immunofluorescent staining and confocal analyses, acetone-fixed frozen sections were washed for 5 min in PBS and incubated for 30 min with the first primary Ab. The slides were then washed in PBS for 5 min. Next, sections were incubated for 30 min with biotinylated secondary Ab, washed, and incubated with Alexa Fluor 488-conjugated streptavidin (Molecular Probes) (1/800) for 20–30 min. After washing, sections were incubated for 30 min with the second primary Ab, washed again, and incubated for 30 min with biotinylated secondary Ab. After washing, sections were incubated with Alexa Fluor 594-conjugated streptavidin (Molecular Probes) (1/800) for 20–30 min and then washed. Cross-reaction between the first secondary Ab and Alexa Fluor 594 was prevented by saturation of all its binding sites with Alexa Fluor 488. Slides were mounted with Vectashield medium (Vector Laboratories) and examined with a Zeiss LSM 510 Meta laser scanning confocal microscope (Zeiss).

Cells

TUBO r-p185$^+$ cells are a cloned cell line from a carcinoma arising in a BALB-neuT mouse (28), whereas r-p185$^+$ 3T3-NKB cells are from BALB/c 3T3 fibroblasts stably cotransfected with the wild-type *r-HER-2*, mouse class I H-2K^d and B7.1 genes (24). Cells were cultured in DMEM (BioWhittaker Europe) supplemented with 20% FBS (Invitrogen Life Technologies).

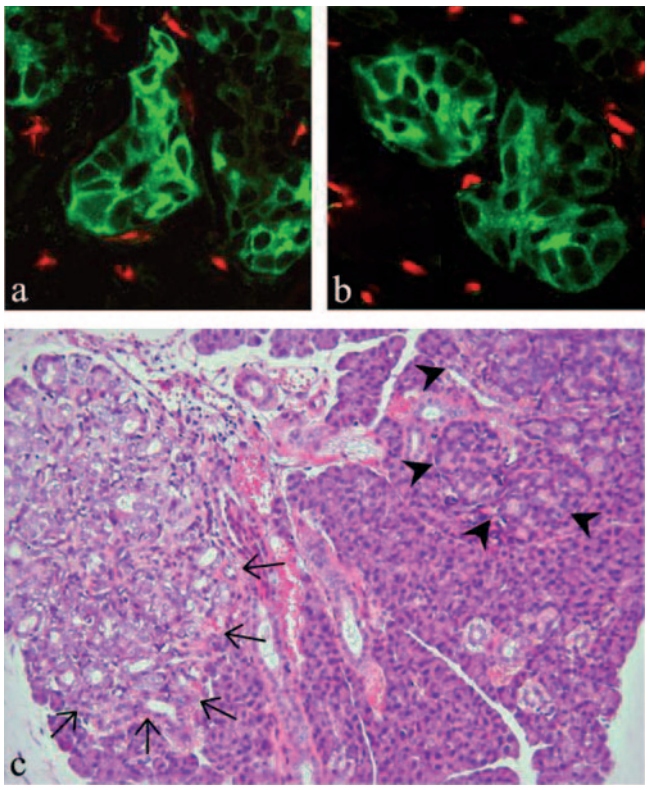

FIGURE 1. Onset of ductal and acinar hyperplasia in the parotid glands of BALB-neuT/p53^{172R-H} mice. At 3 wk of age, large cells expressing r-p185 (in green) become visible in some ducts (*a*) and acini (*b*) of the well vascularized (red-stained microvessels) parotid glands. At 4 wk, these cells gave rise to intercalated ductal (*c*, arrows) and acinar (*c*, arrowheads) hyperplasia consisting in a nodular assembly of ductal structures with a central lumen lined by a single layer of epithelial cells. Acinar hyperplasia is characterized by enlarged acini packed with polygonal cells with granular cytoplasm and hyperchromatic dense nuclei. *a* and *b*, ×1000; *c*, ×200.

Ab response

Sera were collected at week 9, 20, and 40 from individual mice that had received two EC-TM plasmid electroporations and four systemic administrations of IL-12. After 1/100 dilution in PBS-azide-BSA (Sigma-Aldrich), the presence of anti r-p185 Abs was determined by flow cytometry using 3T3-NKB cells. FITC-conjugated goat anti-mouse Ab specific for mouse IgG Fc (DakoCytomation) was use to detect bound primary Ab. For control, we used a normal mouse serum. mAb Ab4 (Oncogene Research Products), which recognizes the EC domain of r-p185, was used as a positive control. After washing, cells were resuspended in PBS-azide-BSA containing 1 mg/ml propidium iodide to remove dead cells and evaluated in a CyAn ADP (DakoCytomation). Serial Ab4 dilutions were used to generate a standard curve to determine the concentration (μg/ml) of anti r-p185, as previously described in detail (24). For isotype determinations, individual sera from three 16-wk-old mice were diluted 1/20 and incubated with BALB/c 3T3 and BALB/c 3T3-NKB. Cells were then incubated for 30 min with rat biotin-conjugated Ab anti-mouse IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 (Caltag Laboratories). After washing, cells were incubated with 5 μl of streptavidin-PE (DakoCytomation) and resuspended in PBS-azide-BSA containing 1 mg/ml propidium iodide to gate dead cells. Flow cytometry was performed with a CyAn ADP (DakoCytomation).

In vitro and in vivo cytotoxicity assays

Fresh or restimulated spleen cells (Spc) were assayed in a 48-h [^{3}H]TdR release assay at E:T from 50:1 to 6:1 in round-bottom, 96-well microtiter plates in triplicate as previously described in detail (29). Spc were restimulated in vitro by culturing for 6 days 1×10^7 cells with 5×10^5 mitomycin C (Sigma-Aldrich)-treated r-p185$^+$ TUBO or 3T3-NKB cells in the presence of 10 U/ml rIL-2 (Eurocetus). In some experiments, Spc were pulsed for 48 h with BrdU labeling solution at 37°C in 5% CO_2 with 5 or 2.5 μg/ml of the r-p185$_{63-71}$ (TYVPANASL) (INBIOS Srl; Biotech Products). This peptide is predicted to bind the H-2^d glycoproteins with high affinity (⟨www.syfpeithi.de/⟩). It was assayed for amino acid composition, HPLC homogeneity, and mass spectrometry weight, dissolved in PBS, aliquoted at 1 mg/ml, and stored frozen at −20°C until used. BrdU uptake was detected with the cell proliferation ELISA BrdU kit (Roche Diagnostic).

In vivo cytotoxicity assay was performed as described by Ritchie et al. (30), with slight modifications. Briefly, a single-cell suspension of 10^7 naive Spc/ml were labeled with two different concentration (0.5 or 5.0 μM) of the fluorescent dye CFSE (Molecular Probes). Spc labeled with 5 μM were also pulsed with r-p185$_{63-71}$ 9-mer peptide with H-2K^d restriction element (31) for 1 h at room temperature. The two Spc populations were mixed together in equal amounts and injected i.v. into three control and treated mice. Mice were sacrificed 48 h later, and single-cell suspensions from spleens were processed individually to evaluate the presence of CFSE high and low cells by CyAn ADP (DakoCytomation) after adding propidium iodine to exclude dead cells. The specific cytolytic activity was calculated as: 100 × (percentage CFSE^{low} cells − percentage CFSE^{high} cells)/percentage CFSE^{low} cells.

Results

Progression of carcinogenesis in parotid glands

A few strongly r-p185$^+$ epithelial cells were initially evident in the intercalated ducts and acini of the parotid glands of 3-wk-old BALB-neuT/p53^{172R-H} mice. One week later, they also expressed the PCNA and gave rise to multiple foci of typical ductal and acinar hyperplasia (Fig. 1). During the fifth week, the acinar hyperplasia shrank, whereas the typical ductal areas expanded and became atypical (Fig. 2, *a–c*). In the seventh week, the parotid glands of most mice displayed multifocal in situ carcinomas wherein the ducts were replaced by discrete, rounded, solid aggregates of r-p185$^+$ and PCNA$^+$ neoplastic epithelial cells with no lumens or fenestrations (Fig. 2, *d–f*). From the eighth week onward, these areas merged to form poorly differentiated larger tumors. Tumor progression was paralleled by a progressive reduction in r-p185 expression that was almost undetectable in the cells of carcinomas of 10-wk-old mice. By contrast, positivity remained high and widely distributed (Fig. 2, *g–i*). The lung metastases observable from the 10th wk were also r-p185 negative. Progressive disappearance of r-p185 expression unaccompanied by a parallel reduction in PCNA expression was never found in p-185$^+$ carcinomas arising in BALB-neuT mice (12, 28, 29, 32). After the 10th wk, the parotids also displayed lesions in dissimilar progression stages wherein progressive diminution of p-185 expression from the more to the less differentiated stages was evident (Fig. 2, *j–l*). This tumor progression occurred earlier in the males. At 4 wk of age, in fact, their multifocal hyperplasia was substantially more advanced and wider, while typical hyperplasia had already progressed to atypical hyperplasia and in situ carcinomas were present in ~50% 1 wk later (data not shown).

The network of small vessels and capillaries supplying the hyperplastic areas was slightly more developed and irregular than

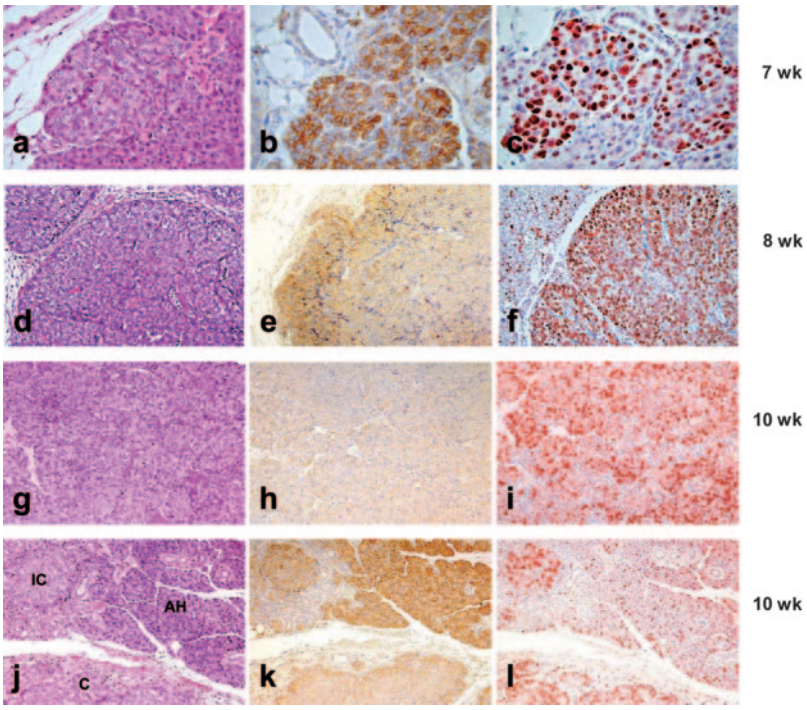

FIGURE 2. Features of in situ and advanced carcinoma developing in the parotid glands of BALB-neuT/p53^{172R-H} mice. Parotid glands of 7-wk-old mice (*left panels*) display foci of in situ carcinoma (*a*) formed of nests of epithelial cells with clear eosinophilic cytoplasm and enlarged nuclei, the majority of which overexpress r-p185 (in brown, *b*) on their cell membrane, and actively proliferate, as shown by nuclear PCNA positivity (in black-red, *c*). Around wk 8, these lesions progressed toward a poorly differentiated carcinoma consisting of large, round to polygonal cells with large and pleomorphic nuclei (*d*) that overexpress r-p185, particularly at the edges of tumor lesions (*e*), where actively proliferating cells are concentrated (*f*). At 10 wk of age, in the poorly differentiated large tumors (*g*), p185 expression is almost absent (*h*), whereas the nuclear PCNA positivity is widely distributed (*i*). After the 10th wk, tumor lesions at different stages were present (*j*). Their p185 expression gradually diminished from the more to the less differentiated stages (*k*), and there was a broad positivity for PCNA (*l*). *a–c*, ×400; *d–l*; ×200.

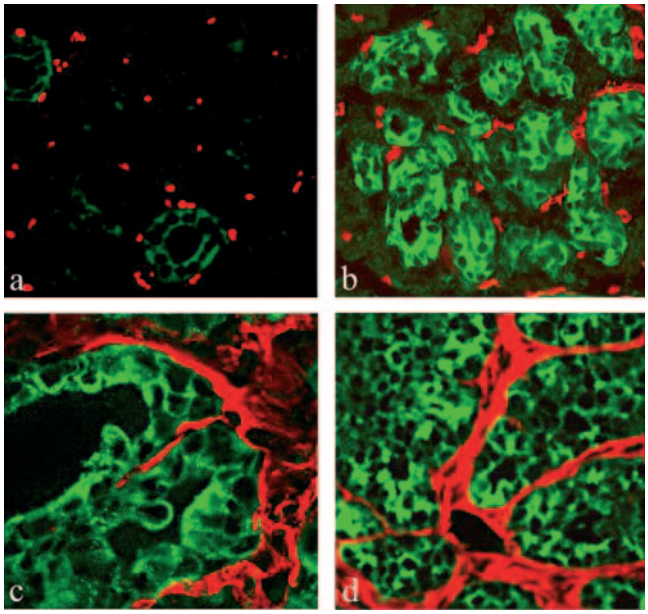

FIGURE 3. Microvessel distribution and tenascin-C production during the progression of hyperplasia to advanced carcinoma in the parotid glands of BALB-p53^{172R-H} mice. Microvessel network (in red) and r-p185 expression (in green) in normal parotid gland, hyperplastic gland (*b* and *c*), and in poorly differentiated (advanced) carcinoma (*d*). The network is well represented in the normal parotid gland, where r-p185 expression is absent (*a*). It twins the small hyperplastic ducts and is more developed inside the hyperplastic areas formed by r-p185 cells. The onset of hyperplasia is associated with a marked production of tenascin-C (in red), which accumulates beside to r-p185-expressing (in green) cells along the basement membranes (c); tenascin-C abundantly lines highly r-p185^{+} neoplastic nests and vessels in the poorly differentiated carcinoma (*d*). *a* and *b*, × 400; *c*, ×1000; and *d*, ×600.

that of the normal parotid gland of BALB/c mice or the healthy parotid tissue of BALB-neuT/p53^{172R-H} mice (Fig. 3, *a* and *b*). The vascular network inside the frankly carcinomatous areas was smaller than that of normal and hyperplastic glands but composed of larger, thicker-walled vessels. Laser scanning confocal analyses revealed that the appearance of PCNA^{+} and r-p185 $^{+}$ cells in the parotid lesions was accompanied by a strong production of tenascin-C, a member of a large family of extracellular matrix proteins whose expression is associated with a migratory or invasive tumor cell phenotype (33, 34). Tenascin-C accumulated along the basement membrane lining hyperplastic and neoplastic aggregates and the related microvessels (Fig. 3, *c* and *d*).

In addition, 3-wk-old BALB-neuT/p53^{172R-H} females began to display multifocal hyperlastic foci in their mammary glands. These progressed to in situ lobular carcinomas and invasive carcinomas in the few mice left alive after the 14th wk of age. This progression was similar to that described in BALB-neuT mice. Most mice succumbed during the 11th and 12th wk of age due to overgrowth of the poorly differentiated and invasive parotid carcinoma and its lung metastases.

Prevention of carcinomas

As previous work had shown that DNA vaccination combined with IL-12's antiangiogenic and immunomodulatory activity slows the progression of mammary lesions in BALB-neuT mice and markedly potentiates anti-HER-2 immunity (24), we determined whether this combination is also effective against the very fast progression of the parotid lesions of BALB-neuT/p53^{172R-H} mice in which p53 had lost its normal function. Therefore, throughout their life, these mice received courses of four weekly i.p. injections of 100 ng of IL-12, followed by a 3-wk rest, together with i.m. electroporation of EC-TM plasmids during the first and third week of each course.

In this preventive setting, the first IL-12 injection was administered at the fifth week of age, when the parotid glands display multiple typical hyperplastic foci that already overexpress r-p185 (Fig. 4).

All untreated mice and all those who received IL-12 or either empty or EC-TM plasmids alone developed bilateral lethal parotid carcinomas (Fig. 5*a*), although only a single tumor appeared or tumor multiplicity was significantly reduced when EC-TM plasmids were used without IL-12. Moreover, >70% of mice that received the combination courses were tumor-free at wk 40 when the experiment ended (Fig. 5*b*). All five female mice and 50% (three of six) of the male mice were tumor-free (Fig. 5*b*). Despite such small numbers, this difference points to a sex-linked discrepancy in the protection afforded by the combined treatment.

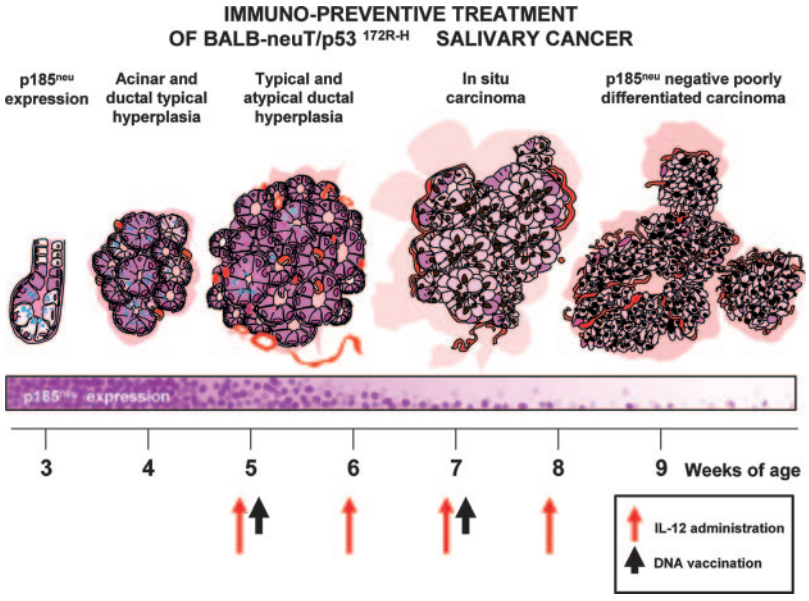

FIGURE 4. Progression of parotid carcinogenesis in untreated BALB-neuT/p53^{172R-H} mice. Indication of the time of i.p. IL-12 administration (red arrows) and DNA vaccination through electroporation (black arrows). In the surviving mice, IL-12 administration, DNA vaccination, or their combination was repeated after 3 wk of rest until the end of the experiment or the death of mice.

a

Stage of parotid lesions when the treatment was started	Treatment	Tumor free/ total mice	Tumor Multiplicity
	None	0/19 (0%)	2.00[a] (38/19)
Diffuse typical and atypical hyperplasia (5 week)	IL-12	0/10 (0%)	2.00 (20/10)
Diffuse typical and atypical hyperplasia (5 week)	Empty plasmid electroporation	0/10 (0%)	2.00 (20/10)
Diffuse typical and atypical hyperplasia (5 week)	EC-TM plasmid electroporation	0/10 (0%)	1.20 (12/10)[c]
Diffuse typical and atypical hyperplasia (5 week)	IL-12 and EC-TM plasmid electroporation	8/11 (73%)[b]	0.27 (3/11)[c, e]
Multifocal in situ carcinomas (7 week)	IL-12	0/ 8 (0%)	2.00 (16/ 8)
Multifocal in situ carcinomas (7 week)	EC-TM plasmid electroporation	0/10 (0%)	1.40 (14/10)[d]
Multifocal in situ carcinomas (7 week)	IL-12 and EC-TM plasmid electroporation	7/24 (29%)[c]	1.00 (24/24)[c]

[a] Number of tumors / mouse at the time of death. A tumor was evident in the left and right salivary glands of all control mice. In brackets is shown the cumulative number of tumors / total treated mice.
[b, c, d] Significantly different ([b] p<0.001; [c] p< 0.0002, [d] p< 0.005) from corresponding values in untreated mice.
[e] Significantly different (p<0.0001) from corresponding values in mice receiving EC-TM plasmids only at week 5.

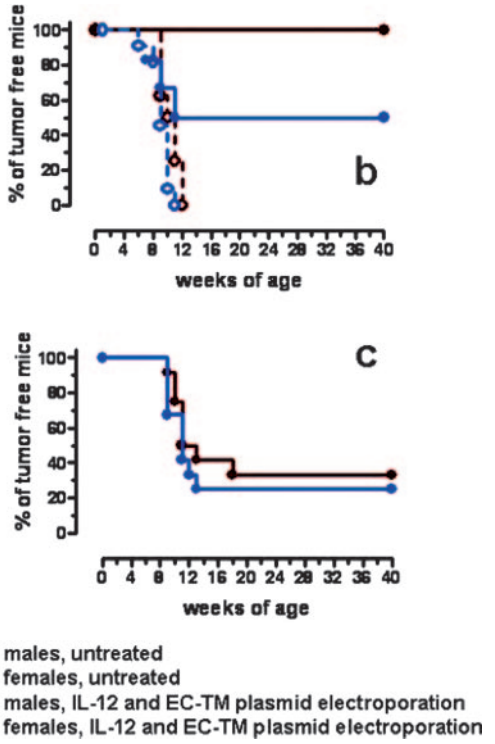

FIGURE 5. Ability of IL-12 in combination with EC-TM plasmid electroporation to prevent the progression of atypical hyperplasia and cure in situ parotid carcinomas. Tumor multiplicity and cure in BALB-p53^{172R-H} mice following the various treatments started at 5 or 7 wk of age (*a*). When male and female mice were evaluated separately, a significantly better protection ($p < 0.0001$) is evident in females treated starting at 5 wk of age (*b*). When the treatment was started at 7 wk, this difference disappeared (*c*).

Pathological findings associated with carcinoma prevention

Histological inspection of the parotid glands of 9-wk-old mice, i.e., 1 wk after the end of the first course (see Fig. 4), showed that several reactive cells had infiltrated the stroma. Epithelial cell ghosts and hemosiderin accumulations were the outcome of microinflammation and hemorrhage (Fig. 6*a*). Histological examination of tumor-free mice at wk 20 (4 wk after the end of the second course) disclosed normal parotid glands (Fig. 6*b*) free from r-p185$^+$ and PCNA$^+$ cells (Fig. 6*c*), a normal microvessel density (Fig. 6*d*) and tenascin-C lining only normal striated and intercalated ducts (Fig. 6*e*).

Cure of HER-2 carcinomas

The remarkable ability of this combination to block parotid carcinogenesis and prevent carcinoma onset prompted us to assess its ability to cure initial and actively proliferating in situ carcinomas. All BALB-neuT/p53^{172R-H} mice receiving electroporations alone starting from wk 7 developed parotid carcinomas, even though tumor multiplicity was reduced (Fig. 5*a*). By contrast, ~30% of mice (25% of males and 33% of females) receiving the combination from wk 7 onward was tumor-free at wk 40 (Fig. 5*c*). Their parotid glands were absolutely normal and free from r-p185$^+$ and PCNA$^+$ cells. Postinflammatory fibrotic foci were absent or very scanty. The lower preventive efficacy of the combination was probably due to the earlier appearance of r-p185$^+$ cells and the faster progression of parotid carcinogenesis in male as opposed to female mice.

Immune events associated with vaccination

High titers of anti r-p185 Abs were present in the sera of mice after the first course (Fig. 7*a*), whereas significantly lower titers were found when electroporation was given alone and no Abs were observed in untreated mice and those receiving IL-12 or empty plasmids only. At wk 16, anti-r-p185 Abs were of the IgG subclasses, with IgG2a as the most represented (Fig. 7*b*). Spc collected at 9, 16, and 40 wk of age from mice receiving the combination, whether freshly isolated or recovered from 6-day cultures with mitomycin-C-inactivated r-p185$^+$ TUBO and 3T3-NKB cells, displayed a marginal cytotoxicity against both TUBO and 3T3-NKB targets (data not shown). Marginal or absent cytotoxic responses were observed when the assays were performed earlier and later. As the dominant r-p185 epitope recognized by CTL in BALB/c mice has been defined (31), it was possible to assess both the cytotoxic response triggered in vivo (Fig. 7*c*) and the proliferative response of Spc in vitro (Fig. 7*d*). While nontolerant wild-type BALB/c female mice treated from wk 5 with IL-12 and DNA vaccine developed a marked cytolytic response to target Spc pulsed with r-p185$_{63–71}$ (TYVPANASL) peptide with H-2K^d restriction element, no significant cytotoxicity was evident in transgenic BALB-neuT/p53^{172R-H} females (Fig. 7*b*). This lack of cytotoxic response in vitro and in vivo fits in well with the reduced proliferative response of Spc from BALB-neuT/p53^{172R-H}-vaccinated mice, as compared with wild-type BALB/c-vaccinated mice, when incubated with the TYVPANASL peptide as evaluated 1 wk after the first course of IL-12 and DNA vaccine (Fig. 7*c*).

Discussion

Present data show, probably for the first time, that the combination of DNA vaccine electroporation with systemic IL-12 administration effectively and sustainedly prevents the onset of parotid carcinomas in most genetically susceptible BALB-neuT/p53^{172R-H} mice and cures their actively proliferating multifocal in situ parotid carcinomas in ~30%. These results extend previous demonstrations of the ability of this combination to protect against both a

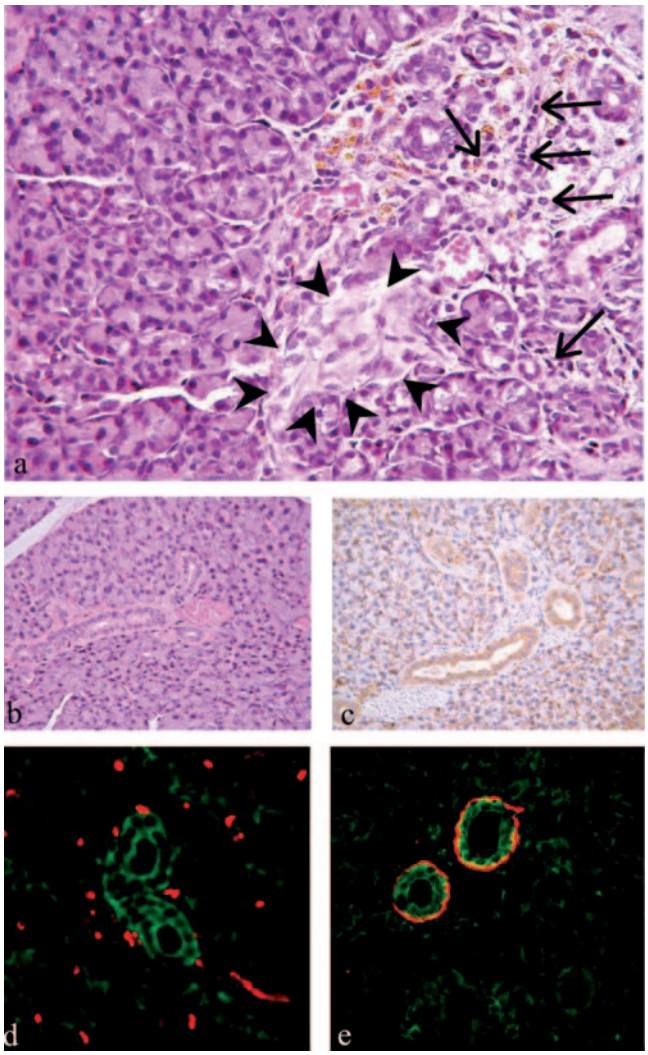

FIGURE 6. Features associated with tumor prevention by the "early" vaccination plus IL-12 treatment in female BALB-neuT/p53^{172R-H} mice. Parotid gland of 9-wk-old treated mice (*a*) showing reactive cells (arrows) penetrating the stroma and in tight contact with ductal and acinar cells ongoing destruction (arrowheads). Hemosiderin accumulation (orange deposits) suggests that microvessel hemorrhage also occurred. At 20 wk of age, treated healthy mice showed histologically normal parotid glands (*b*) that do not stain for r-p185 (*c*) and whose microvessel density was similar to that in the normal gland (*d*, red stained), whereas tenascin-C (*e*, red stained) only lined normal striated ducts. The negative green staining in *d* and *e* indicates the absence of r-p185$^+$ cells. *a*–*e*, x×400.

slowly progressing *HER-2* mammary carcinogenesis (12, 24, 35) and a fast and devastating model of parotid carcinogenesis whose earlier stages are characterized by r-p185 overexpression that decreases as tumor progresses.

In BALB-neuT/p53^{172R-H} mice, fast and lethal parotid carcinogenesis stems from an association of *HER-2* oncogene activation with *p53* oncosuppressor gene functional inactivation. In its early stages, combination of DNA vaccine electroporation with systemic IL-12 is highly effective, whereas electroporation and IL-12 alone are ineffective. As r-p185 overexpression is markedly evident during the early parotid hyperplasia and decreases during the passage from in situ to invasive carcinoma, the combination becomes less effective. A similar progressive disappearance of r-p185 expression unaccompanied by a parallel reduction in PCNA expression was never observed in p-185$^+$ carcinomas arising in BALB-neuT mice. In these mice, r-p185 is equally overexpressed in preneoplastic lesions, invasive cancer, and metastatic cells (12, 28, 29, 32). During the progression of the parotid lesions in BALB-neuT/p53^{172R-H} mice, PCNA cell positivity remains widely distributed and thus becomes progressively less coupled with r-p185 expression. This suggests that the p53 deficiency promotes genetic instability and favors the emergence of tumor clones whose growth becomes independent from *HER-2* overexpression (36–38).

The double requirement of *HER-2* oncogene activation and p53 inactivation makes several features of BALB-neuT/p53^{172R-H} oncogenesis akin to that in humans (39). Most human salivary gland carcinomas, especially the more aggressive forms, are associated with amplification of the *HER-2* oncogene and overexpression of its product. Overexpression and functional impairment of p53 have been noted in most salivary polymorphous low-grade carcinomas, squamous cell carcinomas, and carcinomas arising in benign lesions, such as pleomorphic parotid adenomas (14–16), where the appearance of cells with nuclear p53 accumulation is regarded as the originating precancerous lesion (16).

In a preventive setting, DNA vaccine electroporation combined with systemic IL-12 administration started on wk 5 blocked the neoplastic progression of hyperplastic and preneoplastic lesions in 70% of mice (100% of the females and 50% of the males). When it was started at wk 7 to cure tumors, it was successful in ~30% of mice of both sexes. These results show that this combination is an extremely effective way of both blocking the progression of and destroying r-p185$^+$ cells accumulated in typical and atypical hyperplasia foci but has a limited ability to cure in situ carcinomas. The preventive treatment was less effective in males, probably because areas of r-p185-negative carcinoma were already present, whereas in females of the same age there were only typical and atypical hyperplasia foci and carcinomatous lesions appeared later. The earlier development of such foci in males, coupled with the presence of carcinoma as early as the fifth week, is in line with the observation that in rodents the development of the salivary ducts, which in BALB-neuT/p53^{172R-H} mice contain the most r-p185 expressing cells, is under multihormonal regulation and larger in males (40).

The curative potential of IL-12 in combination with DNA vaccine was associated with a marked anti-r-p185 Ab response in the absence of a significant CD8 T cell-mediated cytotoxicity. This absence is not surprising, because *HER-2* transgenic mice express r-HER-2 in the thymus, and central and peripheral tolerance takes place (41, 42). Spectratyping (43) showed that central tolerance in transgenic BALB-neuT and BALB-neuT/p53^{172R-H} mice mainly acts by silencing the CD8$^+$ component of the TCR repertoire, although an high avidity CD4$^+$ T cell response escapes tolerance induction (S. Rolla, F. Cavallo, and F. Ria, manuscript in preparation). Induction of a high titer of Abs appears to account for both the prevention and cure of fast growing and early metastasizing salivary carcinomas. These provocative findings are germane to similar results observed in mice transgenic for *HER-2* oncogene that undergo mammary carcinogenesis, which show that vaccine-induced inhibition of *HER-2* carcinogenesis mostly, if not solely, rests on the elicitation of a high titer of anti-HER-2 Abs (26, 28, 44–46). By hampering r-p185's ability to form homo- and heterodimers that spontaneously transduce proliferative signals (47) and by stripping r-p185 from the cell membrane and causing its internalization in the cytoplasm (48–50), Abs inhibit the cell signaling properties of r-p185 and block the proliferative ability of HER-2$^+$ tumor cells (11, 29, 44). Anti-r-p185 Abs also affect tumor growth by mediating Ab-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (51).

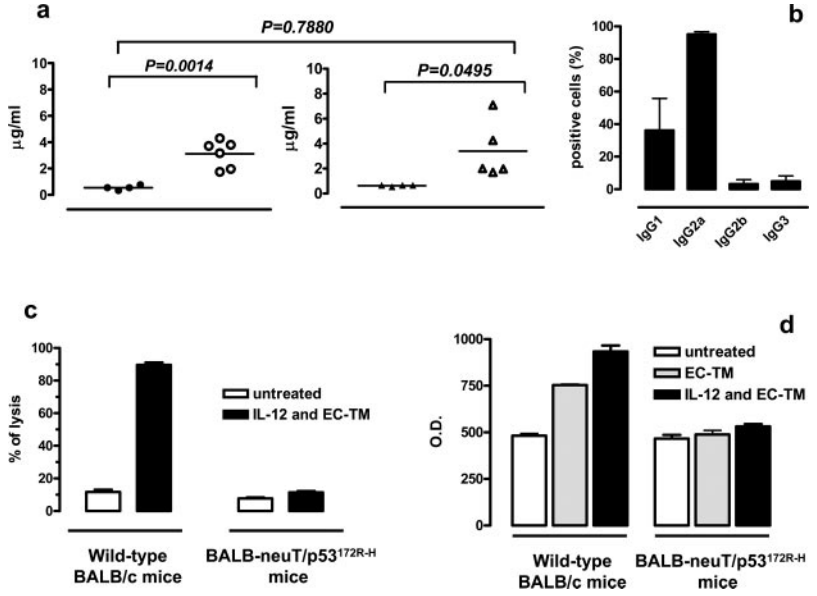

FIGURE 7. Anti r-p185 cellular and Ab response in BALB-neuT/p53^{172R-H} mice. *a*, Anti-r-p185 Ab production by BALB-neuT/p53^{172R-H} mice. Sera collected at wk 9 from individual mice that had received two EC-TM plasmid electroporations and four systemic administrations of IL-12 were tested by indirect immunofluorescence, followed by cytometry. Values in untreated males (▲) and females (●) and in IL-12-treated and EC-TM plasmid electroporated males (△) and females (○). Black lines show the cohort geometric mean. Isotypes of anti-r-p185 Abs (*b*) and anti-r-p185 CTL response (*c*) in 16-wk-old BALB-neuT/p53^{172R-H} mice treated with systemic IL-12 in combination with EC-TM plasmid electroporation courses starting at wk 5. *b*, Percentage of BALB/c 3T3-NKB cells stained by the isotypes of anti-r-p185 Abs. IgA and IgM were not detectable. *c*, Sixteen-week -old untreated mice or mice treated with systemic IL-12 in combination with EC-TM plasmid electroporation courses starting at wk 5 were given i.v. CFSE-labeled Spc pulsed or not with r-p185$_{63–71}$ (TYVPANASL) 9mer peptide. Forty-eight hours later, single-cell suspensions from spleens processed individually were analyzed by CyAn ADP. Histograms showing the mean ± SD of the cytotoxic activity detected in single animals in at least three animals are shown. *d*, Fresh Spc from variously treated BALB/c and BALB-neuT/p53^{172R-H} mice were pulsed for 48 h with BrdU labeling solution at 37°C in 5% CO_2 with 2.5 µg/ml of the r-p185$_{63–71}$ peptide. Results are shown as BrdU uptake, detected with the cell proliferation ELISA BrdU kit (Roche Diagnostic), and measured as OD.

In the mammary lesions of BALB-neuT mice, anti-r-p185 Abs induced by the vaccine and IL-12 combination down-regulate r-p185 on the cell membrane of mammary lesions paralleled by a marked decrease of tumor cell proliferation and a marked recruitment of reactive cells in and around the preneoplastic lesions frequently surrounded by fibrotic tissue (24). In BALB-neuT/p53^{172R-H} mice, the inflammatory infiltrate and postinflammatory fibrosis were scanty or absent. Only mice treated at 7–9 wk displayed minute areas of fibrosis, probably the outcome of prior inflammation. In most tumor-free mice, the parotid was fully normal and free from r-p185$^+$ and PCNA$^+$ cells. The absence of morphological indicators of inflammation or tissue necrosis indicated that the proliferative blockade and/or destruction of r-p185$^+$ cells caused by the combination must have taken place in a discrete manner without leaving the slightest trace as soon as these cells appeared in the ducts or acini, or were still dispersed in multiple hyperplasia foci since their massive destruction when grouped together in a single solid tumor mass would have led to inflammation and reparative fibrosis. These patterns are strongly suggestive of a direct role of anti-r-p185 Ab on tumor cell proliferation and survival. The sustained presence of high titers of anti-r-p185 Ab leads to selective removal of cells forming the neoplastic lesions and elimination of the continuously reappearing r-p185 neoplastic cells, as occurs in mice in which the transgene is embedded in the genome.

While IL-12's enhancement of the specific protective response elicited by DNA vaccine electroporation fits in well with its powerful adjuvant activity in a variety of vaccines against infectious diseases (52–54), the curative potential of the combination appears to be also the outcome of IL-12's ability to hamper early carcinogenesis by inhibiting *HER-2* tumor angiogenesis (34, 55). Present data suggest that IL-12's antiangiogenic activity contributed to the success of the vaccination by inhibiting the growth and progression of the hyperplasia, since the vascular network supporting such growth was slightly more developed than that of the normal gland and composed of small, delicate, and newly formed capillaries. These findings are in accord with our previous finding that the antiangiogenic activity of systemic IL-12 is very effective on those fragile capillary sprouts, which accompany the shift from the preneoplastic to the neoplastic condition. After the transition from hyperplasia to invasive carcinoma, the lower sensitivity of mature and differentiated blood vessels tapers off the efficacy of IL-12's antiangiogenic activity (12, 25, 35).

In the successfully treated mice, the complete disappearance of hyperplasia foci was also illustrated by the absence of tenascin-C, a hexameric extracellular matrix glycoprotein expressed during embryonic development and re-expressed in remodeling and proliferative processes, such as wound healing and tumorigenesis. In human salivary gland tumors, tenascin-C is mostly observed in less differentiated and more highly malignant tumors, such as solid carcinomas. Therefore, its absence in the glands of the treated mice suggests that both proliferation and remodeling following the destruction of r-p185$^+$ had been abolished.

We have previously documented that in male BALB-neuT mice expression of the *r-HER-2* oncogene product in the ducts and acini of the parotid gland results in typical (at 4–5 wk of age) and then atypical (from the 6th to the 18th wk) hyperplasia preceding the development of cancers from the confluence of multiple ductal hyperplastic foci, whereas hyperplastic acini behave as an abortive preneoplastic lesion (13). Development of cancer was thus preceded by a long period marked by hyperplasia alone. During this time span, the salivary gland cells overexpressing the activated

r-HER-2 oncogene product probably underwent further gene changes inducing the transformation of typical and atypical hyperplasia into r-p185^{+} carcinoma. By contrast, 8-wk-old male and female BALB-neuT/p53$^{172R\text{-}H}$ mice developed p185-negative poorly differentiated and invasive parotid carcinomas that were much more aggressive and less differentiated than the acinar cell carcinomas previously observed in older male BALB-neuT mice. Morphological examination showed that development of their hyperplastic and preneoplastic lesions occurred from the fourth to the seventh week. This model with its identification of the seventh to the eighth week as the time of transition to a r-p185-expression-independent poorly differentiated cancer illustrates both the potential and the limits of vaccination and permits a direct assessment of the potential of a timely vaccine.

Greater and long-lasting protection can still be conferred against even highly genetically unstable tumors provided vaccination begins before genetic instability becomes coupled with an intense tumor cell proliferation that permits the expansion of r-p185-negative escaping clones. Tumor cells in the preneoplastic lesions of BALB-neuT/p53$^{172R\text{-}H}$ mice are potentially very unstable from the beginning. However, in the absence of a significant proliferation, these cells do not give rise to a significant population of escaping cells, and the vaccine provides a major protection. Fewer mice are protected, and less efficient induction of immunity and a lower efficacy of effector mechanisms are evident if vaccination begins when advanced multifocal lesions are present.

In conclusion, these results provide a direct endorsement of the notion that vaccines may provide a sustained inhibition of precancerous lesions even if they fail to control invasive cancer (56, 57). Therefore, the message for human disease is neither to refrain from vaccinating patients with Li-Fraumeni syndrome, nor that due to tumor intrinsic genetic instability there is little point in vaccinating against a disappearing Ag. Instead, present data suggest that a vaccine may be able to convey a significant and long-lasting protection against indolent precancerous lesions, even when a mutated *p53* enables the early development of a population of Ag-negative potentially aggressive tumor cells. The next key questions to be addressed concern the possibility of extending the efficacy of vaccination to cope with more advanced neoplastic stages. Vaccination against multiple target Ags expressed by preneoplastic lesions (58) may perhaps reduce the likelihood of an escape from immune attack. However, while recent data on cancer progression have shifted the onset of genetic instability to the early phases of the natural history of tumors, the accumulation of genetic "hits" leading to cancer progression is a function of time (59). The low proliferation rate of indolent preneoplastic lesions cuts down the likelihood of selection of immune-resistant clones, whereas the vaccine-alerted immune response may inhibit the lesion before complete neoplastic transformation takes place. Because preventive vaccines operate during the early phases of carcinogenesis, effective inhibition of r-p185^{+} cells arrests the whole process and renders the selection of r-p185-loss variants unlikely (60). Translation of these preclinical observations into a novel preventive treatment in cancer is a far-distant prospect. Even so, our results provide a preclinical picture of ability of vaccines to hampering early stages of carcinogenesis.

Disclosures

The authors have no financial conflict of interest.

References

1. Speight, P. M., and A. W. Barrett. 2002. Salivary gland tumours. *Oral Dis.* 8: 229–240.
2. Sessions, R. B., L. B. Harrison, and A. A. Forastiere. 2001. Tumors of the salivary glands and paraganglioma. In *Cancer: Principles and Practice of Oncology.* V. T. De Vita, Jr., S. Hellman, and S. A. Rosenberg, eds. Lippincott Williams & Wilkins, Philadelphia, pp. 886–906.
3. Gooden, E., I. J. Witterick, D. Hacker, I. B. Rosen, and J. L. Freeman. 2002. Parotid gland tumours in 255 consecutive patients: Mount Sinai Hospital's quality assurance review. *J. Otolaryngol.* 31: 351–354.
4. Skalova, A., Starek, V. Kucerova, P. Szepe, and L. Plank. 2001. Salivary duct carcinoma—a highly aggressive salivary gland tumor with HER-2/neu oncoprotein overexpression. *Pathol. Res. Pract.* 197: 621–626.
5. Nguyen, L. H., M. J. Black, M. Hier, P. Chauvin, and L. Rochon. 2003. HER2/neu and Ki-67 as prognostic indicators in mucoepidermoid carcinoma of salivary glands. *J. Otolaryngol.* 32: 328–331.
6. Skalova, A., I. Starek, T. Vanecek, V. Kucerova, L. Plank, P. Szepe, S. Di Palma, and I. Leivo. 2003. Expression of *HER-2/neu* gene and protein in salivary duct carcinomas of parotid gland as revealed by fluorescence in-situ hybridization and immunohistochemistry. *Histopathology* 42: 348–356.
7. Etges, A., D. S. Pinto, Jr., L. P. Kowalski, F. A. Soares, and V. C. Araujo. 2003. Salivary duct carcinoma: immunohistochemical profile of an aggressive salivary gland tumour. *J. Clin. Pathol.* 56: 914–918.
8. Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science* 235: 177–182.
9. Kokai, Y., J. N. Myers, T. Wada, V. I. Brown, C. M. LeVea, J. G. Davis, K. Dobashi, and M. I. Greene. 1989. Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts. *Cell* 58: 287–292.
10. Di Fiore, P. P., O. Segatto, W. G. Taylor, S. A. Aaronson, and J. H. Pierce. 1990. EGF receptor and erbB-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. *Science* 248: 79–83.
11. Yarden, Y., and M. X. Sliwkowski. 2001. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* 2: 127–137.
12. Boggio, K., G. Nicoletti, E. Di Carlo, F. Cavallo, L. Landuzzi, C. Melani, M. Giovarelli, I. Rossi, P. Nanni, C. De Giovanni, et al. 1998. Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of *Her-2/neu* transgenic mice. *J. Exp. Med.* 188: 589–596.
13. Diodoro, M. G., E. Di Carlo, R. Zappacosta, M. Iezzi, A. Coletti, A. Modesti, T. D'Antuono, G. Forni, and P. Musiani. 2000. Salivary carcinoma in *HER-2/neu* transgenic male mice: an angiogenic switch is not required for tumor onset and progression. *Int. J. Cancer* 88: 329–335.
14. Nordkvist, A., E. Roijer, G. Bang, H. Gustafsson, M. Behrendt, W. Ryd, S. Thoresen, K. Donath, and G. Stenman. 2000. Expression and mutation patterns of *p53* in benign and malignant salivary gland tumors. *Int. J. Oncol.* 16: 477–483.
15. Ohki, K., H. Kumamoto, R. Ichinohasama, M. Suzuki, T. Yamaguchi, S. Echigo, K. Motegi, and K. Ooya. 2001. Genetic analysis of DNA microsatellite loci in salivary gland tumours: comparison with immunohistochemical detection of hMSH2 and p53 proteins. *Int. J. Oral Maxillofac. Surg.* 30: 538–544.
16. Ohtake, S., J. Cheng, H. Ida, M. Suzuki, K. Ohshiro, W. Zhang, and T. Saku. 2002. Precancerous foci in pleomorphic adenoma of the salivary gland: recognition of focal carcinoma and atypical tumor cells by P53 immunohistochemistry. *J. Oral Pathol. Med.* 31: 590–597.
17. Horak, E., K. Smith, L. Bromley, S. LeJeune, M. Greenall, D. Lane, and A. L. Harris. 1991. Mutant p53, EGF receptor and c-erbB-2 expression in human breast cancer. *Oncogene* 6: 2277–2284.
18. Croci, S., G. Nicoletti, L. Landuzzi, C. De Giovanni, A. Astolfi, C. Marini, E. Di Carlo, P. Musiani, G. Forni, P. Nanni, and P. L. Lollini. 2004. Immunological prevention of a multigene cancer syndrome. *Cancer Res.* 64: 8428–8434.
19. Akashi, M., and H. P. Koeffler. 1998. Li-Fraumeni syndrome and the role of the *p53* tumor suppressor gene in cancer susceptibility. *Clin. Obstet. Gynecol.* 41: 172–199.
20. Attardi, L. D., and T. Jacks. 1999. The role of p53 in tumour suppression: lessons from mouse models. *Cell Mol. Life Sci.* 55: 48–63.
21. Li, B., K. L. Murphy, R. Laucirica, F. Kittrell, D. Medina, and J. M. Rosen. 1998. A transgenic mouse model for mammary carcinogenesis. *Oncogene* 16: 997–1007.
22. Olive, K. P., D. A. Tuveson, Z. C. Ruhe, B. Yin, N. A. Willis, R. T. Bronson, D. Crowley, and T. Jacks. 2004. Mutant *p53* gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* 119: 847–860.
23. Lang, G. A., T. Iwakuma, Y. A. Suh, G. Liu, V. A. Rao, J. M. Parant, Y. A. Valentin-Vega, T. Terzian, L. C. Caldwell, L. C. Strong, et al. 2004. Gain of function of a *p53* hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell* 119: 861–872.
24. Spadaro, M., E. Ambrosino, M. Iezzi, E. Di Carlo, P. Sacchetti, C. Curcio, A. Amici, W. Z. Wei, P. Musiani, P. L. Lollini, et al. 2005. Cure of mammary carcinomas in *Her-2* transgenic mice through sequential stimulation of innate (neoadjuvant interleukin-12) and adaptive (DNA vaccine electroporation) immunity. *Clin. Cancer Res.* 11: 1941–1952.
25. Boggio, K., E. Di Carlo, S. Rovero, F. Cavallo, E. Quaglino, P. L. Lollini, P. Nanni, G. Nicoletti, S. Wolf, P. Musiani, and G. Forni. 2000. Ability of systemic interleukin-12 to hamper progressive stages of mammary carcinogenesis in HER2/neu transgenic mice. *Cancer Res.* 60: 359–364.
26. Rovero, S., A. Amici, E. D. Carlo, R. Bei, P. Nanni, E. Quaglino, P. Porcedda, K. Boggio, A. Smorlesi, P. L. Lollini, et al. 2000. DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. *J. Immunol.* 165: 5133–5142.
27. Di Carlo, E., S. Rovero, K. Boggio, E. Quaglino, A. Amici, A. Smorlesi, G. Forni, and P. Musiani. 2001. Inhibition of mammary carcinogenesis by systemic interleukin 12 or p185neu DNA vaccination in *Her-2/neu* transgenic BALB/c mice. *Clin. Cancer Res.* 7: 830s–837s.

28. Quaglino, E., S. Rolla, M. Iezzi, M. Spadaro, P. Musiani, C. De Giovanni, P. L. Lollini, S. Lanzardo, G. Forni, R. Sanges, et al. 2004. Concordant morphologic and gene expression data show that a vaccine halts HER-2/neu preneoplastic lesions. *J. Clin. Invest.* 113: 709–717.
29. Quaglino, E., M. Iezzi, C. Mastini, A. Amici, F. Pericle, E. Di Carlo, S. M. Pupa, C. De Giovanni, M. Spadaro, C. Curcio, et al. 2004. Electroporated DNA vaccine clears away multifocal mammary carcinomas in *HER-2/neu* transgenic mice. *Cancer Res.* 64: 2858–2864.
30. Ritchie, D. S., I. F. Hermans, J. M. Lumsden, C. B. Scanga, J. M. Roberts, J. Yang, R. A. Kemp, and F. Ronchese. 2000. Dendritic cell elimination as an assay of cytotoxic T lymphocyte activity in vivo. *J. Immunol. Methods* 246: 109–117.
31. Nagata, Y., R. Furugen, A. Hiasa, H. Ikeda, N. Ohta, K. Furukawa, H. Nakamura, K. Furukawa, T. Kanematsu, and H. Shiku. 1997. Peptides derived from a wild-type murine proto-oncogene *c-erbB-2/HER2/neu* can induce CTL and tumor suppression in syngeneic hosts. *J. Immunol.* 159: 1336–1343.
32. Pannellini, T., G. Forni, and P. Musiani. 2004. Immunobiology of *HER-2/neu* transgenic mice. *Breast Dis.* 20: 33–42.
33. Tsunoda, T., H. Inada, I. Kalembeyi, K. Imanaka-Yoshida, M. Sakakibara, R. Okada, K. Katsuta, T. Sakakura, Y. Majima, and T. Yoshida. 2003. Involvement of large tenascin-C splice variants in breast cancer progression. *Am. J. Pathol.* 162: 1857–1867.
34. Jahkola, T., T. Toivonen, I. Virtanen, K. von Smitten, S. Nordling, K. von Boguslawski, C. Haglund, H. Nevanlinna, and C. Blomqvist. 1998. Tenascin-C expression in invasion border of early breast cancer: a predictor of local and distant recurrence. *Br. J. Cancer* 78: 1507–1513.
35. Cifaldi, L., E. Quaglino, E. Di Carlo, P. Musiani, M. Spadaro, P. L. Lollini, S. Wolf, K. Boggio, G. Forni, and F. Cavallo. 2001. A light, nontoxic interleukin 12 protocol inhibits *HER-2/neu* mammary carcinogenesis in BALB/c transgenic mice with established hyperplasia. *Cancer Res.* 61: 2809–2812.
36. Podsypanina, K., Y. Li, and H. E. Varmus. 2004. Evolution of somatic mutations in mammary tumors in transgenic mice is influenced by the inherited genotype. *BMC Med.* 2: 24.
37. Liu, S., W. Liu, J. L. Jakubczak, G. L. Erexson, K. R. Tindall, R. Chan, W. J. Muller, S. Adhya, S. Garges, and G. Merlino. 2002. Genetic instability favoring transversions associated with ErbB2-induced mammary tumorigenesis. *Proc. Natl. Acad. Sci. USA* 99: 3770–3775.
38. Huang, G. C., S. Hobbs, M. Walton, and R. J. Epstein. 2002. Dominant negative knockout of *p53* abolishes ErbB2-dependent apoptosis and permits growth acceleration in human breast cancer cells. *Br. J. Cancer* 86: 1104–1109.
39. Hanahan, D., and R. A. Weinberg. 2000. The hallmarks of cancer. *Cell* 100: 57–70.
40. Pinkstaff, C. A. 1998. Salivary gland sexual dimorphism: a brief review. *Eur. J. Morphol.* 36 Suppl: 31–34.
41. Reilly, R. T., M. B. Gottlieb, A. M. Ercolini, J. P. Machiels, C. E. Kane, F. I. Okoye, W. J. Muller, K. H. Dixon, and E. M. Jaffee. 2000. HER-2/neu is a tumor rejection target in tolerized *HER-2/neu* transgenic mice. *Cancer Res.* 60: 3569–3576.
42. Ercolini, A. M., B. H. Ladle, E. A. Manning, L. W. Pfannenstiel, T. D. Armstrong, J. P. Machiels, J. G. Bieler, L. A. Emens, R. T. Reilly, and E. M. Jaffee. 2005. Recruitment of latent pools of high-avidity $CD8^+$ T cells to the antitumor immune response. *J. Exp. Med.* 201: 1591–1602.
43. Ria, F., P. van den Elzen, L. T. Madakamutil, J. E. Miller, E. Maverakis, and E. E. Sercarz. 2001. Molecular characterization of the T cell repertoire using immunoscope analysis and its possible implementation in clinical practice. *Curr. Mol. Med.* 1: 297–304.
44. Park, J. M., M. Terabe, Y. Sakai, J. Munasinghe, G. Forni, J. C. Morris, and J. A. Berzofsky. 2005. Early role of $CD4^+$ Th1 cells and antibodies in *HER-2* adenovirus vaccine protection against autochthonous mammary carcinomas. *J. Immunol.* 174: 4228–4236.
45. Sakai, Y., B. J. Morrison, J. D. Burke, J. M. Park, M. Terabe, J. E. Janik, G. Forni, J. A. Berzofsky, and J. C. Morris. 2004. Vaccination by genetically modified dendritic cells expressing a truncated neu oncogene prevents development of breast cancer in transgenic mice. *Cancer Res.* 64: 8022–8028.
46. Nanni, P., L. Landuzzi, G. Nicoletti, C. De Giovanni, I. Rossi, S. Croci, A. Astolfi, M. Iezzi, E. Di Carlo, P. Musiani, et al. 2004. Immunoprevention of mammary carcinoma in *HER-2/neu* transgenic mice is IFN-γ and B cell dependent. *J. Immunol.* 173: 2288–2296.
47. Klapper, L. N., N. Vaisman, E. Hurwitz, R. Pinkas-Kramarski, Y. Yarden, and M. Sela. 1997. A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. *Oncogene* 14: 2099–2109.
48. Katsumata, M., T. Okudaira, A. Samanta, D. P. Clark, J. A. Drebin, P. Jolicoeur, and M. I. Greene. 1995. Prevention of breast tumour development in vivo by downregulation of the p185neu receptor. *Nat. Med.* 1: 644–648.
49. Yip, Y. L., and R. L. Ward. 2002. Anti-ErbB-2 monoclonal antibodies and ErbB-2-directed vaccines. *Cancer Immunol. Immunother.* 50: 569–587.
50. Klapper, L. N., H. Waterman, M. Sela, and Y. Yarden. 2000. Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res.* 60: 3384–3388.
51. Sliwkowski, M. X., J. A. Lofgren, G. D. Lewis, T. E. Hotaling, B. M. Fendly, and J. A. Fox. 1999. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). *Semin. Oncol.* 26: 60–70.
52. Chattergoon, M. A., V. Saulino, J. P. Shames, J. Stein, L. J. Montaner, and D. B. Weiner. 2004. Co-immunization with plasmid IL-12 generates a strong T-cell memory response in mice. *Vaccine* 22: 1744–1750.
53. Cui, F. D., H. Asada, M. L. Jin, T. Kishida, M. Shin-Ya, T. Nakaya, M. Kita, M. Ishii, M. Iwai, T. Okanoue, et al. 2005. Cytokine genetic adjuvant facilitates prophylactic intravascular DNA vaccine against acute and latent herpes simplex virus infection in mice. *Gene Ther.* 12: 160–168.
54. Cooper, D., M. W. Pride, M. Guo, M. Cutler, J. C. Mester, F. Nasar, J. She, V. Souza, L. York, E. Mishkin, et al. 2004. Interleukin-12 redirects murine immune responses to soluble or aluminum phosphate adsorbed HSV-2 glycoprotein D towards Th1 and $CD4^+$ CTL responses. *Vaccine* 23: 236–246.
55. Cavallo, F., E. Quaglino, L. Cifaldi, E. Di Carlo, A. Andre, P. Bernabei, P. Musiani, G. Forni, and R. A. Calogero. 2001. Interleukin 12-activated lymphocytes influence tumor genetic programs. *Cancer Res.* 61: 3518–3523.
56. Ye, X., J. McCarrick, L. Jewett, and B. B. Knowles. 1994. Timely immunization subverts the development of peripheral nonresponsiveness and suppresses tumor development in simian virus 40 tumor antigen-transgenic mice. *Proc. Natl. Acad. Sci. USA* 91: 3916–3920.
57. Forni, G., P. L. Lollini, P. Musiani, and M. P. Colombo. 2000. Immunoprevention of cancer: is the time ripe? *Cancer Res.* 60: 2571–2575.
58. Finn, O. J. 2003. Premalignant lesions as targets for cancer vaccines. *J. Exp. Med.* 198: 1623–1626.
59. Vogelstein, B., and K. W. Kinzler. 2004. Cancer genes and the pathways they control. *Nat. Med.* 10: 789–799.
60. Lollini, P. L., F. Cavallo, P. Nanni, and G. Forni. 2006. Vaccines for tumour prevention. *Nat. Rev. Cancer*. 6: 204–216.