BMC Medicine

BioMed Central

Research article

Evolution of somatic mutations in mammary tumors in transgenic mice is influenced by the inherited genotype

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Published: 15 June 2004

BMC Medicine 2004, **2**:24 doi:10.1186/1741-7015-2-24

Received: 18 February 2004
Accepted: 15 June 2004

This article is available from: http://www.biomedcentral.com/1741-7015/2/24

Abstract

Background: *MMTV-Wnt1* transgenic mice develop mammary hyperplasia early in development, followed by the appearance of solitary mammary tumors with a high proportion of cells expressing early lineage markers and many myoepithelial cells. The occurrence of tumors is accelerated in experiments that activate *FGF* proto-oncogenes or remove the tumor suppressor genes *Pten* or *P53*, implying that secondary oncogenic events are required for progression from mammary hyperplasia to carcinoma. It is not known, however, which oncogenic pathways contribute to *Wnt1*-induced tumorigenesis – further experimental manipulation of these mice is needed. Secondary events also appear to be required for mammary tumorigenesis in *MMTV-Neu* transgenic mice because the transgene in the tumors usually contains an acquired mutation that activates the Neu protein-tyrosine kinase.

Methods: cDNA or DNA from the mammary glands and mammary tumors from *MMTV-Wnt1*, *MMTV-Wnt1/p53*$^{-/-}$, *MMTV-Neu* transgenic mice, and newly generated *MMTV-Wnt1/MMTV-Neu* bitransgenic mice, was sequenced to seek activating mutations in *H-Ras*, *K-Ras*, and *N-Ras* genes, or in the *MMTV-Neu* transgene. In addition, tumors from bitransgenic animals were examined to determine the cellular phenotype.

Results: We found activating mutations at codons 12, 13, and 61 of *H-Ras* in just over half of the mammary tumors in *MMTV-Wnt1* transgenic mice, and we confirmed the high frequency of activating mutations of *Neu* in tumors in *MMTV-Neu* transgenic mice. Tumors appeared earlier in bitransgenic *MMTV-Wnt1/MMTV-Neu mice*, but no *Ras* or *MMTV-Neu* mutations were found in these tumors, which were phenotypically similar to those arising in *MMTV-Wnt1* mice. In addition, no *Ras* mutations were found in the mammary tumors that arise in *MMTV-Wnt1* transgenic mice lacking an intact *P53* gene.

Conclusions: Tumorigenic properties of cells undergoing functionally significant secondary mutations in *H-Ras* or the *MMTV-Neu* transgene allow selection of those cells in *MMTV-Wnt1* and *MMTV-Neu* transgenic mice, respectively. Alternative sources of oncogenic potential, such as a second transgenic oncogene or deficiency of a tumor suppressor gene, can obviate the selective power of those secondary mutations. These observations are consistent with the notion that somatic evolution of mouse mammary tumors is influenced by the specific nature of the inherited cancer-promoting genotype.

Background

Several transgenic mouse models have been generated in efforts to identify or confirm genes that can participate in breast carcinogenesis and to study the pathogenic process (for review, see [1]). In general, these models have revealed that a wide variety of proto-oncogenes can initiate mammary tumorigenesis when expressed under the control of an appropriate promoter (usually the mouse mammary tumor virus long terminal repeat (MMTV LTR) or the whey acidic protein (WAP) transcriptional control domain). They have also revealed that tumorigenesis is a stochastic process, resulting in the appearance of solitary tumors in one or a few of the 10 mammary glands several months after birth, and that secondary mutational events, inherited deficiencies in tumor suppressor genes, or a combination of transgenes can accelerate the onset of tumor growth. In addition, persistent tumor growth generally requires the continued expression of the initiating oncogene, unless certain secondary mutations have occurred to render the tumor independent of the transgenic oncogene [2-4].

Despite these generalizations, the characteristics of mammary tumors arising in transgenic mice differ in a fashion characteristic of the initiating oncogene. For example, the expression profiles of tumors reflect the transgenic oncogene [5]; moreover, those tumors induced by genes that activate the Ras signaling pathway (for example, polyoma middle T antigen, *ErbB2/Neu*, and *H-Ras*) are more similar to each other than to tumors induced by components of the Wnt signaling pathway (*Wnt1*, *β-catenin*, and *c-Myc*) with respect both to gene expression patterns ([5], S. Huang *et al*., in preparation) and to cellular composition and histology [6-9]. In tumors induced by components of the Wnt signaling pathway, a high proportion of cells produce proteins (Sca-1, Keratin 6) associated with early stages of mammary development, whereas few such cells are observed in tumors induced by activators of Ras signaling [9]. In addition, the presence of many tumor cells with myoepithelial features (for example, expression of smooth muscle actin) and other observations suggest that in the *Wnt* pathway oncogenes transform cells positioned early in the developmental program for mammary tissue, whereas oncogenes like *ErbB2/Neu* that activate *Ras* proteins, either transform cells that are more advanced developmentally or drive cells to a more mature epithelial character during oncogenesis.

Efforts to understand these complex pathogenic events must take into consideration the secondary oncogenic events that are presumed to be required to convert one or a few of the many mammary cells expressing a transgenic oncogene into a frankly tumorigenic cell. Several findings offer clues to the nature of such secondary events. For example, the appearance of mammary tumors in *MMTV-Wnt1* transgenic mice can be accelerated by a second transgene (*MMTV-Fgf3*, [10]), by proviral insertion mutations in *Fgf* genes [11-13], and by inherited deficiencies of *P53* or *Pten* [14,15]. Similarly, tumors appear earlier in *MMTV-c-Myc/MMTV-v-H-Ras* bitransgenic mice than in monotransgenic mice [16], and, in *c-Myc* transgenic mice, tumors that do not require continued expression of *c-Myc* usually contain somatically mutated *K-Ras2* or *N-Ras* genes [3]. When tumors occur in animals that inherit a transgene encoding normal ErbB2/Neu protein, the tumors usually exhibit a secondary somatic mutation of *ErbB2/Neu* that stimulates the protein-tyrosine kinase activity of the gene product [17].

In this report, we have attempted to identify additional somatic events that can promote progression to a tumorigenic phenotype in *MMTV-Wnt1* transgenic mice by seeking acquired mutations in *Ras*. In addition, we have asked whether accelerating factors, such as inheritance of a second transgenic oncogene, *MMTV-Neu*, or deficiency in a tumor suppressor gene, *p53*, diminishes selection for cells that have acquired potentially oncogenic mutations. In the course of these studies, we were also able to determine that the phenotypic properties of tumors arising in *MMTV-Wnt1/MMTV-Neu* bitransgenic mice resemble those of tumors induced by Wnt rather than Ras signaling.

Methods

Mice and tissues

MMTV-Wnt1 (FVB/NJ-Tg [Wnt1] 1 Hev/J), *MMTV-Neu* (FVB/N-Tg [MMTVNeu] 202 Mul/J), and *p53*$^{-/-}$ (129-Trp53tm1Tyj/J) mice were purchased from Jackson Labs (Bar Harbor, ME, USA) and maintained or backcrossed for many generations on the FVB background. All mice were maintained in a specific pathogen-free facility on a standard diet.

RNA, DNA, and protein extraction from tumors

Mouse mammary tumors were collected at the time of necropsy or tumor extraction surgery and snap frozen in liquid nitrogen. Frozen tumors were ground in liquid nitrogen and the resulting powder was placed in three tubes containing appropriate buffers. For RNA extraction, about 50 mg of tumor powder was dissolved in Trizol (#15596-018; Invitrogen, Carlsbad, CA, USA), followed by phenol-chloroform extraction and ethanol precipitation. For DNA extraction, about 50 mg of tumor powder was digested overnight with 2 mg/ml Proteinase K (#1 373 200; Roche, Indianapolis, IN, USA) in 20 mM Tris, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, pH 8.5 buffer, followed by phenol-chloroform extraction and ethanol precipitation. For protein extraction, about 50 mg of tumor powder was dissolved in 20 mM Tris, 150 mM NaCl, 1% Triton X, 2 mM EDTA, 1x protease inhibitor cocktail (complete tablets, #1 697 498, Roche), 1 mM Na_3VO_4, 40

mM NaF, incubated on ice for up to 30 min and cleared by centrifugation.

Some DNA samples from *MMTV-Wnt1* tumors with wild type, heterozygous or null *p53* allele (generated by L. Donehower [18]) on a mixed genetic background were a gift from Larry Donehower (Baylor College of Medicine).

Ras *and* MMTV-Neu *cDNA synthesis*

Extracted RNA was copied with gene-specific primers to make cDNA, which was then amplified with the appropriate primers using the SuperScript™ One-Step RT-PCR Kit (#10928-034; Invitrogen) according to manufacturer's instructions. For mouse *K-Ras* and *N-Ras* amplification, primer sequences and cycling conditions were kindly provided by Lewis Chodosh and Robert Boxer (University of Pennsylvania School of Medicine). Mouse *H-Ras* codons 3–99 were amplified either with primers generated based on the sequences provided by Chodosh and Boxer, or with an additional reverse primer spanning exons 2 and 3: HARAS.B2: 5'GATCTGCTCCCTGTACTGATGG3'.

MMTV-Neu transgene cDNA was amplified with primers AB2913 and AB1310 [17], which amplify the region corresponding to nucleotides 1492–2117 of rat *Neu* cDNA.

Amplification products were purified with QIAquick PCR Purification Kit (#28106; Qiagen, Valencia, CA, USA) according to manufacturer's instructions.

H-Ras *DNA synthesis*

Tumor DNA was extracted as described above, and extracted DNA was amplified with Taq polymerase (#N808-0152; Roche) in 1 × PCR Buffer (#N808-0006; Roche), 2.5 mM dNTPs in the presence of the following primers: mouse *H-Ras* exon 1: HRAS.F1A: 5'-CCTTGGCTAAGTGTGCTTC-3', HRAS.B1A: 5'-CCACCTCTGGCAGGTAG-3'; mouse *H-Ras* exon 2: HRAS.F2A: 5'-GGATTCTCTGGTCTGAGG3-', HRAS.2B2: 5'-GGATATGAGCCAGCTAGC-3'. Amplification was performed for 30 cycles of 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. Amplification products were purified as above.

Sequencing

All sequencing was carried out by the Memorial Sloan-Kettering Cancer Center (MSKCC) Sequencing Core Facility. Every cDNA was sequenced with both forward and reverse primers used for amplification, and some samples were also sequenced with the nested reverse primer. Sequencing peaks were inspected for overall integrity and legibility. The sample was scored as mutant when heterozygous peaks at positions 12,13, 59, and 61 were at least the same height as the wild type peaks in at least one sequencing direction. Sequences were also downloaded into Vector NTI 5.2.5 (InforMax, Inc., Frederick, MD, USA) program and aligned on the Baylor College of Medicine Search Launcher [19] against the sequences deposited in GenBank (mouse *H-Ras*: nm008284; mouse *K-Ras:* xm110615; mouse *N-Ras*: nm010937, rat *Neu*: X03362), to detect mutations (or lack of thereof) in the transcripts.

Immunoblotting and immunohistochemistry

Proteins were extracted from tumors as described above, and lysates were diluted 1:1 with 2 × sample buffer (#LC2676, Invitrogen) with 10% β-mercapthoethanol (#M-3148, Sigma, St. Louis, MO, USA), boiled, loaded on Novex SDS/PAGE gels (#EC6075BOX; Invitrogen) and separated by electrophoresis for 1.5 hrs at 125 V and then stained with Coomassie Blue to estimate protein concentrations. Equal amounts of tumor lysates were then reloaded on the 10% SDS/PAGE gels and separated by electrophoresis. Separated proteins were transferred onto nitrocellulose membranes according to the gel manufacturer's instructions. Membranes were incubated for 1 hour with 5% solution of non-fat dried milk (Carnation, Nestlé, Solon, OH, USA), followed by 1 hour incubation with rabbit polyclonal antibodies against phospho-ERK, 1:500 dilution, (#9101S; Cell Signaling, Beverly, MA, USA), total ERK, 1:500 dilution, (#9102; Cell Signaling) or mouse monoclonal antibody against γ-tubulin, 1:1,000 dilution, (GTU88; Sigma), followed by appropriate secondary antibodies conjugated with HRP (donkey-anti-rabbit, 1:10,000, #111-035-144, Jackson Immunochemicals, West Grove, PA, USA; or rat-anti-mouse kappa light chain, 1:2,000, #2067 1323, Zymed, San Francisco, CA, USA).

Tissues were fixed in 10% buffered formalin (#SF100-4, Fisher, Fair Lawn, NJ, USA) for 16–24 hrs, transferred to 70% ethanol, and shipped to be paraffin-embedded and sectioned at Histoserv (Germantown, MD, USA). Individual sections were deparaffinized, rehydrated and boiled in 1 mM EDTA for antigen retrieval. Slides were then treated with peroxidase, followed by incubation with the appropriate blocking serum from the Vectastain kits (anti-rat-ABC-peroxidase, #PK6104; anti-rabbit-ABC-peroxidase, # PK6101; MOM, # PK2200, Vector Laboratories, Inc., Burlingame, CA, USA) according to manufacturer's instructions. Primary antibodies against phospho-ERK (rabbit polyclonal, #9101S; Cell Signaling), keratin 8 (TROMA, rat polyclonal, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), smooth muscle actin (SMA, mouse monoclonal, #M0851; Dako, Carpinteria, CA, USA), or mouse keratin 6 (rabbit polyclonal, #PRB-169P; Covance, Princeton, NJ, USA) were applied for 1 hr, followed by an incubation with biotin-conjugated appropriate secondary antibody and signal amplification according to Vectastain kit manufacturer's instructions (Vector Laboratories, Inc.). Color was developed with NovaRed™ substrate kit (#SK-

4800; Vector Laboratories, Inc.) according to manufacturer's instructions.

Results

Activating mutations in H-Ras *occur in about half of mammary tumors in* MMTV-Wnt1 *mice*

Wnt1-induced mammary tumors contain elevated levels of *c-Myc* RNA and protein, as expected from stimulation of the Wnt signaling pathway [20]. Since an *H-Ras* transgene can hasten tumorigenesis when combined with a *c-Myc* transgene [16] and since *K-Ras* or *N-Ras* is frequently mutated in *c-Myc*-induced tumors [3], we decided to look for secondary somatic mutations in mammary tumors arising in *MMTV-Wnt1* transgenic mice by sequencing cDNA copies of *Ras* mRNAs in the tumors. Mutations were scored only if approximately half of the resulting amplified DNA had the same mutation, implying growth selection of an oncogenic cell in which the observed mutation occurred.

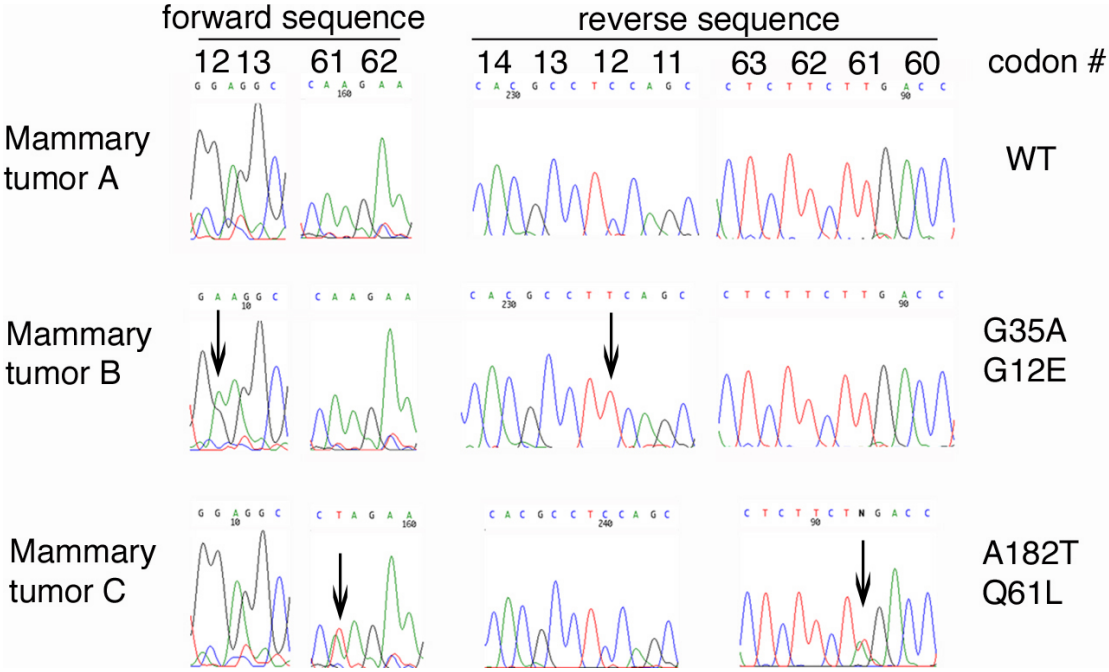

Figure 1
Mutations in the *H-Ras* gene. Representative examples of somatic mutations in the *H-Ras* cDNA from primary mammary tumors of the *MMTV-Wnt1* mice. Sequence chromatograms of codons 12, 13, and 61 are shown as they appear in the forward and reverse sequencing directions. Mutant peaks are indicated by arrows. The nucleotide and amino acid alterations are indicated on the right.

Table 1: Frequency and type of *H-Ras* mutations in *MMTV-Wnt1* tumors

	Codon mutated	Number (% total)
Activating *H-Ras* mutations	CAA61CTA	10 (22%)
	GGA12GAA	5 (11%)
	CAA61CGA	4 (9%)
	CAA61AAA	2 (4%)
	CAA61CAT	1 (2%)
	GGC13AGA	1 (2%)
	GGC13GTT	1 (2%)
H-Ras WT		22 (48%)
Total		46 (100%)

Primary tumor cDNA was amplified and sequenced with *H-Ras*-specific primers at least once in each direction as described in Methods. Products containing mutant peaks equal to or greater in height than the wild type peaks in at least one sequencing direction were scored as mutation-positive. Mutations in codons 12,13, 59, and 61 were scored as activating mutations.

We initially sought activating *Ras* mutations by sequencing codons 3–99 of *H-Ras*, 1–98 of *N-Ras*, and 1–94 of *K-Ras*. All of the sequences contain codons 12, 13, 59, and 61 – sites of the mutations most commonly associated with oncogenic Ras proteins. We found mutations in codons 12 and 61 of *H-Ras*, but not in those of *N-Ras* or *K-Ras* in cDNA from the first 10 tumor samples (Figure 1). We subsequently focused on the *H-Ras* cDNA and found activating mutations in codons 12,13, or 61 in 24 of an additional 36 primary mammary carcinomas (Table 1). We then examined 20 tumors without activating mutations in *H-Ras* for activating point mutations in *K-Ras* and *N-Ras*, but failed to find mutations in these two genes. In addition, we were unable to detect mutant *H-Ras* cDNA made from RNA extracted from hyperplastic mammary glands from *MMTV-Wnt1* transgenic mice ($n = 6$) or from virgin or lactating mammary glands from non-transgenic mice ($n = 6$), implying that, if *H-Ras* mutations occurred before the appearance of primary tumors, an insufficient number of cells in the glands harbored the mutations to allow detection, and that selection during tumorigenic growth would be required.

We made a preliminary effort to define the selectable trait conferred on mammary cells in *MMTV-Wnt1* transgenic mice by an activating mutation in *H-Ras*. Tumors in these mice arise after a latency of similar length, have similar growth characteristics, and metastasize to the lungs with similar frequencies regardless of whether they do or do not contain a mutant *H-Ras* gene (data not shown).

These findings may simply mean that any requirement for activation of the *Ras* pathway in *Wnt1*-induced tumors may be satisfied by some other means, such as other oncogenic mutations; this issue is further addressed in a later section.

We have also used biochemical methods to look for the consequences of the *H-Ras* mutations. For example, the oncogenic activity of mutant Ras proteins in mouse cells is dependent on the Raf kinase pathway, which phosphorylates and activates the ERK1/p44 and ERK2/p42 mitogen-activated protein kinases (MAPKs) [21]. We have compared levels of phospho-ERK in Wnt1-induced tumors with mutant and wild type *H-Ras* genes by protein blotting and immunohistochemistry (Figure 2). While tumors induced by an *MMTV-v-H-Ras* transgene contained high levels of phospho-ERK, *MMTV-Wnt1* and *MMTV-c-Myc*-induced tumors had lower levels, independent of *H-Ras* mutation status (Figure 2A). Similarly, there was no difference in phospho-ERK staining patterns in tumor sections from any of the *MMTV-Wnt1*-induced tumors, although intense staining was observed in *MMTV-v-H-Ras* induced tumors (Figure 2B). Thus we do not know which of the several signaling pathways affected by Ras may be responsible for growth selection of tumor cells containing *H-Ras* mutations.

Mammary tumorigenesis is accelerated in MMTV-Wnt1/MMTV-Neu *bitransgenic mice*

Her-2/Neu acts in part through the Ras signaling pathway [22-24], a feature that may account for the remarkable similarity between the phenotypes of mammary tumors found in *MMTV-Neu* and *MMTV-v-H-Ras* transgenic mice [25]. These observations suggest that an inherited *Neu* transgene might mimic the effects of somatic mutation of a *Ras* gene. If so, tumors might arise earlier in *MMTV-Wnt1/MMTV-Neu* bitransgenic mice than in mice carrying a single transgene, and any cells that acquired *H-Ras* mutations would not have a selective growth advantage; hence *H-Ras* mutations would not be detected in our tests of tumor genotypes. In addition, with bitransgenic mice, we could ask whether a co-existing *MMTV-Wnt1* transgene eliminated the selective advantage conferred by an activating mutation in the coding domain of the *MMTV-Neu* transgene, and we could ask whether the phenotype of tumor cells in resulting tumors resembled the phenotype of tumors induced by components of the Wnt pathway, the Neu/Ras pathway, or both.

Therefore we crossed *MMTV-Wnt1* transgenic mice to a *MMTV-Neu* transgenic line that carries a cDNA encoding normal rat ErbB2/Neu protein [26]. In the bitransgenic *MMTV-Wnt1/MMTV-Neu* progeny, tumors were detected significantly earlier than in sibling females harboring only an *MMTV-Neu* transgene or in *MMTV-Wnt1* transgenic mice (Figure 3). Over fifty percent (8/14) of the female *MMTV-Wnt1/MMTV-Neu* mice developed tumors by 12 weeks of age, twice as early as a similar cohort of *MMTV-Wnt1* mice ($t_{50} = 25$ weeks, $n = 44$), and almost four times as early as the *MMTV-Neu* congenic mice ($t_{50} = 39$ weeks, $n = 26$), maintained in the same facility.

This acceleration of tumor formation demonstrates that *Wnt1* and *Neu* transgenes can cooperate in oncogenic transformation of the mouse mammary gland. To determine whether the presence of an inherited *Neu* transgene obviated the selective advantage of secondary somatic mutations in *H-Ras*, observed in many of the mammary tumors induced by a single *MMTV-Wnt1* transgene (Table 1), we sequenced DNA amplified from *H-Ras* RNA obtained from 11 tumors arising in the *MMTV-Wnt1/MMTV-Neu* bitransgenic animals. None of these samples showed mutations in the first two exons of *H-Ras*, supporting the notion that the presence of a *Neu* transgene provided most or all of the growth advantage attributed to *Ras* mutations in *MMTV-Wnt1* or *MMTV-c-Myc* transgenic mice (Table 1, [3]).

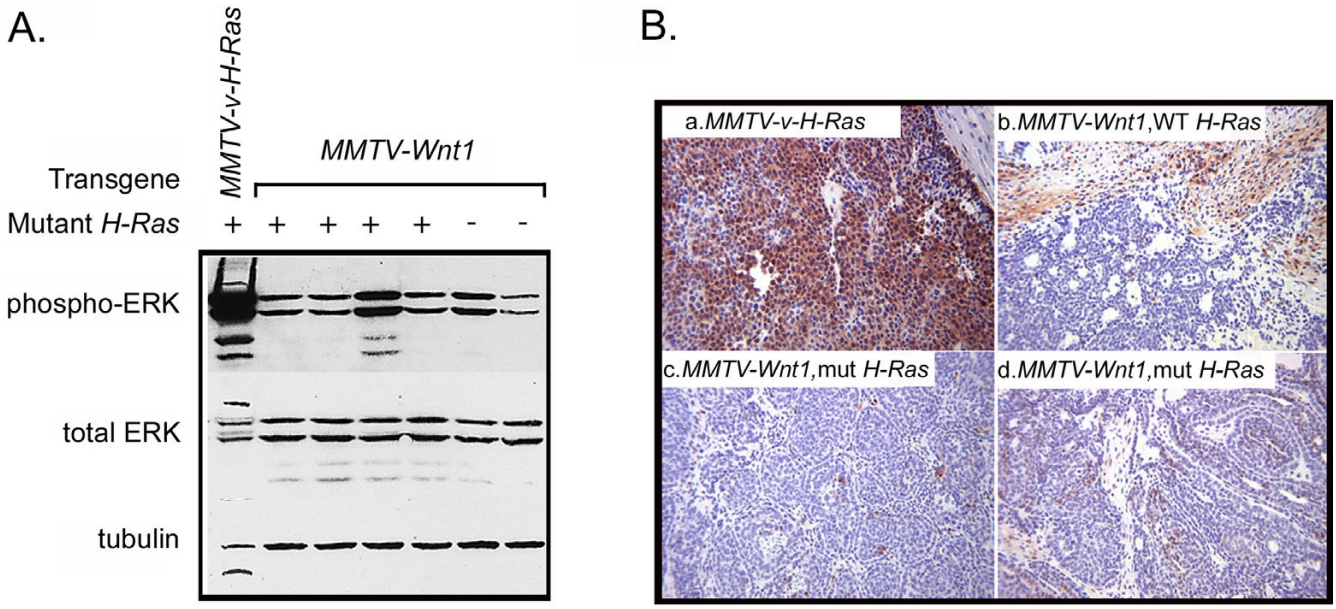

Figure 2
ERK phosphorylation in *MMTV-Wnt1* tumors with and without *H-Ras* mutations. **(A)** Protein lysates from tumors from a *MMTV-v-H-Ras* mouse (first lane) and *MMTV-Wnt1* mice with and without activating *H-Ras* mutations were subjected to electrophoresis in 10% polyacrylamide gels, transferred to a nitrocellulose membrane and then exposed to antibodies against phospho-ERK, total ERK and γ-tubulin as described in Methods. **(B)** Primary tumor sections from (a) *MMTV-v-H-Ras* mouse and (b) *MMTV-Wnt1* mice without activating *H-Ras* mutations and (c,d) with activating *H-Ras* mutations were incubated with antibodies against phospho-ERK as described in Methods.

Mammary tumors reported in the *MMTV-Neu* monotransgenic line used here have usually acquired mutations in the *Neu* coding domain of the transgene, enhancing the enzymatic activity of its product; about 70% of these tumors exhibit point mutations, small deletions, or insertions in the extracellular portion of the receptor in or near the cysteine-rich domain encoded by nucleotides 1492–2117 of rat *Neu* cDNA [17]. However, none of 11 mammary tumors derived from *MMTV-Wnt1/MMTV-Neu* bitransgenic mice had evidence of mutations in the relevant portions of the *Neu* transgene. This result has two implications: that the presence of an inherited *MMTV-Wnt1* transgene strongly diminishes any selective advantage conferred by secondary somatic mutations in the *Neu* transgene, and that expression of a wild-type version of the *Neu* transgene is sufficient to provide the growth advantage that is apparently conferred by secondary somatic mutations of *H-Ras* in *MMTV-Wnt1*-induced tumors.

Activating H-Ras *mutations are not found in* Wnt1-*induced tumors arising in* p53 *null mice*

We have previously shown that deficiency of *p53* dramatically accelerates the appearance of mammary tumors in *MMTV-Wnt1* transgenic mice [14]. More recently, Gunther *et al.* showed that tumors induced by *Wnt1*

Tumors in the MMTV-Wnt1/MMTV-Neu *bitransgenic mice are morphologically similar to* Wnt1*-induced tumors, despite expression of the* Neu *transgene*

MMTV-Wnt1-induced tumors contain multiple mammary cell types, and many cells express early lineage markers, such as Sca-1 and keratin-6 [9]. In contrast, *MMTV-Neu*-induced mammary tumors are composed nearly exclusively of luminal epithelial cells and have a low proportion of Sca-1- and keratin-6-positive cells [9]. Remarkably, all tumors from bitransgenic mice were similar to the *Wnt1*-induced tumors, and not to the *Neu*-induced ones (Figure 4A). We detected multiple cell types within these tumors, including smooth muscle actin-positive myoepithelial cells, keratin-8-positive luminal epithelial cells, and keratin 6-positive cells (Figure 4B, panels a-c). Expression of the *MMTV-Neu* transgene was observed in the luminal epithelial cells from these tumors by immunohistochemistry (Figure 4B, panel d) and by an RT-PCR assay with transgene-specific primers (Figure 4C).

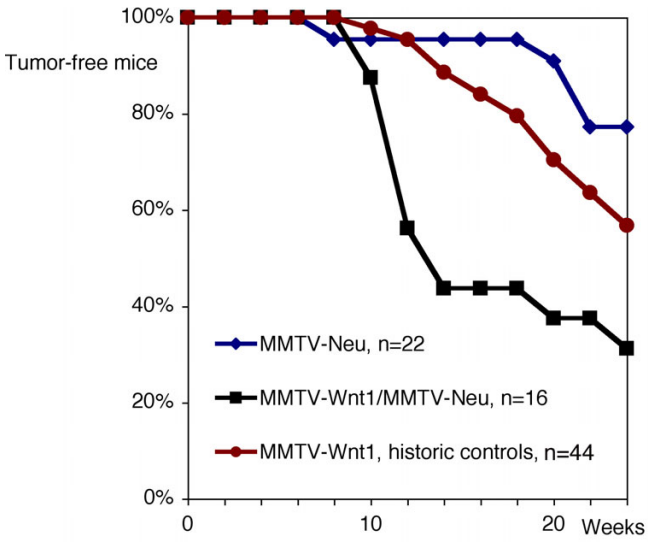

Figure 3
Kaplan-Meiyer plot of tumor incidence in *MMTV-Neu* and *MMTV-Wnt1/MMTV-Neu* siblings and in *MMTV-Wnt1* historical controls. *MMTV-Neu* and *MMTV-Wnt1/MMTV-Neu* female mice generated by crossing *MMTV-Wnt1* male and *MMTV-Neu* female mice were inspected weekly for mammary tumors. The number of *MMTV-Wnt1* females generated in this cross was too low to include in the analysis (n = 4). The rate of appearance of tumors in *MMTV-Wnt1* females in a previously studied cohort maintained under the same conditions is presented instead.

acquire Wnt1 independence in a *p53*-deficient background [2]. We therefore asked whether we could detect *H-Ras* mutations in mammary tumors induced by an *MMTV-Wnt1* transgene in a *p53*-null background; a failure to find such mutations would imply either that p53 is somehow required for such mutations to occur or, more likely, that an inherited deficiency of *p53* conferred a growth advantage to *Wnt1*-expressing mammary cells that would outweigh any selective advantage provided by a secondary somatic mutation in *H-Ras*.

To explore this issue, we examined cDNA or DNA from 16 tumors from *MMTV-Wnt1* transgenic, $p53^{-/-}$ mice and from 11 tumors from *MMTV-Wnt1* transgenic, $p53^{+/-}$ mice, with or without loss of heterozygosity (LOH). *Wnt1*-induced tumors arising on a *p53* heterozygous background, irrespective of the LOH status, contain *H-Ras* mutations at a frequency (5/11) similar to that described above for *Wnt1*-induced tumors on a wild type *p53* background. Importantly, however, no *H-Ras* mutations were found in any of the sixteen *Wnt1*-induced tumors on a $p53^{-/-}$ background ($P < 0.05$). We conclude that an inherited deficiency of *p53* is likely to reduce or eliminate, by an unknown mechanism, any selective growth advantage that might otherwise be contributed by secondary somatic mutations of *H-Ras* in Wnt1-producing mammary cells. The finding of *H-Ras* mutations in the small number of tumors with loss of *p53* heterozygosity implies that *Ras* mutation occurs before *p53* LOH, since *Ras* mutations appear not to confer a selective advantage in *p53* null cells. However, a larger cohort of tumors undergoing LOH at the *p53* locus will be required to establish the conclusion more firmly.

Discussion

Most contemporary accounts of oncogenesis assume that multiple mutations affecting tumor suppressor genes and proto-oncogenes collaborate to convert a normal cell into a cancer cell. When these genetic events occur sequentially by somatic mutations during tumor development, it is assumed that the functionally significant mutations confer a selective advantage, such as an augmented rate of growth, protection from apoptosis, or promotion of further mutations, which explains why most if not all cells in a tumor exhibit multiple acquired mutations, consistent with repeated clonal selection.

In this report, we have used several features of recent studies of breast carcinogenesis in genetically manipulated mice to explore the contributions made to a multi-step oncogenic process by a variety of inherited and somatic mutations. We have identified mutations in the *H-Ras* proto-oncogene as a significant feature of *Wnt1*-induced tumorigenesis (Table 1), and we have shown that an inherited *Neu* transgene or an inherited deficiency of the *p53* tumor suppressor gene can provide a selective advantage to *Wnt1*-expressing mammary cells that overrides the selective advantage conferred by somatic mutation of *H-Ras*. This conclusion is based on our failure to find *H-Ras* mutations in any tumors arising in bitransgenic (*MMTV-Wnt1/MMTV-Neu*) or *p53*-deficient (*MMTV-Wnt1/*$p53^{-/-}$) mice (Table 2).

It is not difficult to reconcile the lack of *H-Ras* mutations in tumors from bitransgenic mice based on the overlapping signaling pathways in which Neu and Ras participate [22,23,27,28] and on the evidence for similarities in gene expression profiles and cell-type composition of mouse mammary tumors induced by these two genes [5,9,24,25]. However, the absence of *H-Ras* mutations in *Wnt1*-induced tumors from *p53* null mice is surprising, since it is well known that an activated *Ras* gene can collaborate with *p53* deficiency to promote both transformation of cultured cells [29] and tumor formation *in vivo*. For example, a mutant *K-Ras* gene induces lung adenocarcinomas more rapidly in *p53*-deficient than in *p53*-wild type mice [30,31], and *K-Ras* mutations are often accompanied by loss of *p53* function in human cancers of the colon,

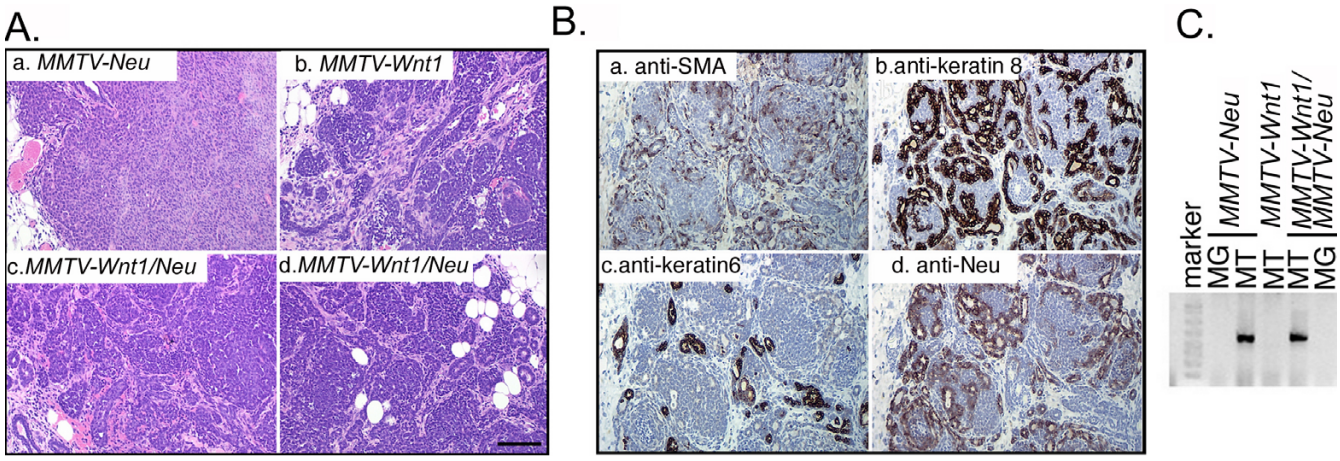

Figure 4
Tumors from *MMTV-Wnt1/MMTV-Neu* mice exhibit morphologic characteristics of *Wnt1*-induced tumors, despite expression of the *Neu* transgene. **(A)** Histological appearance of the tumors from (a) *MMTV-Neu*, (b) *MMTV-Wnt1*, and (c, d) *MMTV-Wnt1/MMTV-Neu* mice. Note solid morphology and scant stroma in the tumor from the *MMTV-Neu* mouse, in contrast with branched ductular architecture and dense stroma of the tumors from *MMTV-Wnt1* and bitransgenic mice. **(B)** Immunohistochemistry on the consecutive sections of the tumor from a bitransgenic mouse with the following mammary lineage markers: (a) anti-smooth muscle actin (SMA), (b) anti-keratin 8 (TROMA), (c) anti-keratin 6, and (d) anti-Her2/Neu marker Ab3, was performed as described in Methods. The Her2/Neu marker is expressed in a subpopulation of cells similar to the one positive for keratin 8. **(C)** Detection of Neu RNA in mammary glands and tumors. RT-PCR for the rat Neu on the total RNA samples from the *MMTV-Neu* mouse mammary gland (MG) and tumor (MT), *MMTV-Wnt1* mouse mammary tumor (MT), and *MMTV-Wnt1/MMTV-Neu* mouse mammary tumor (MT) and gland (MG).

Table 2: Frequency of *H-Ras* mutations in *MMTV-Wnt1/p53*$^{+/-}$, *MMTV-Wnt1/p53*$^{-/-}$, and *MMTV-Wnt1/MMTV-Neu* tumors

	MMTV-Wnt1/p53$^{+/-}$	*MMTV-Wnt1/p53*$^{-/-}$	*MMTV-Wnt1/MMTV-Neu*
With *H-Ras* mutations	5 (2 with *p53* LOH)	0	0
Without *H-Ras* mutations	6 (3 with *p53* LOH)	16	11
Total	11 (5 with *p53* LOH)	16	11

Primary tumor cDNAs (11 *MMTV-Wnt1/p53*$^{-/-}$ samples) or DNAs (5 *MMTV-Wnt1/p53*$^{-/-}$ samples and all of the MMTV-Wnt1/p53$^{+/-}$ samples) were amplified and sequenced with *H-Ras*-specific primers at least once in each direction, as described in Methods. Mutations were scored by the same criteria described in Table 1.

pancreas, and other organs [32-34]. However, effects of inherited mutations on the detection of somatic mutations were previously reported in other mouse models. For example, mammary tumors from *MMTV-TGF-α/MMTV-Neu* and from MMTV-*Neu*/ *p53*^{R172H} bitransgenic mice do not contain somatic mutations in the *Neu* transgene [35,36].

One curious aspect of our findings is the observation of *Ras* mutations affecting only *H-Ras* and never *K-* or *N-Ras* in *Wnt1*-induced mammary tumors. This contrasts sharply with the report by D'Cruz *et al.* [3] of mutations affecting mostly *K-Ras* and also *N-Ras*, but never *H-Ras*, in mouse mammary tumors induced by an *MMTV-c-Myc* transgene. However, three tumors from the small cohort of the *MMTV-c-Myc* mice maintained in our lab were found to carry mutations in *H-Ras*, and one had a mutation in *K-Ras* (KP, unpublished data). We do not have an explanation for these differences in mutation spectrum and do not know whether they reflect differences in mutational rates at *Ras* loci (for example, as a consequence of environmental exposures), differences in the selective advantage conferred by different mutant Ras proteins in cells expressing different oncogenes, or differences in the genetic backgrounds of the mouse lines used.

In the course of these experiments we have also found that tumors from *MMTV-Wnt1/MMTV-Neu* bitransgenic

animals were remarkably similar to those induced in *MMTV-Wnt1* mice, although they appeared much earlier than tumors in monotransgenic animals, indicating cooperative effect of the two transgenes. The dominant effect of *Wnt1* expression in these tumors closely resembles the dominant effect of the *Myc* transgene in tumors from the *MMTV-c-Myc/MMTV-Neu* and *MMTV-c-Myc/MMTV-v-Ha-Ras* bitransgenic mice [6]. This observation suggests that the *Wnt* signaling pathway has a dominant effect over the *Ras* signaling pathway in transformation of mammary epithelial cells. This might be related to the recently described effects of Wnts on stem cell maintenance during normal development [37-40]. Dominance of the Wnt phenotype in this cross might also be explained by a difference in time and level of transgene expression in the mammary tissue.

Conclusions

Selection of somatic oncogenic mutations in mouse mammary tumors depends on the nature of inherited factors: transgenic oncogenes and loss of function mutations in tumor suppressor genes.

Competing interests

None declared.

Authors' contributions

KP planned, analyzed, and presented the experiments described in this paper. KP carried out the *MMTV-Wnt1/MMTV-Neu* cross and YL carried out the *MMTV-Wnt/p53-/-* cross. YL helped to analyze the data and contributed to editing the manuscript. HV helped to plan and oversee the experiments and contributed to the writing and editing of the manuscript.

Acknowledgements

The authors thank Jennifer Doherty, Mary Barrett, and Daisy Chen for expert technical help in mouse colony maintenance and assistance in tumor cDNA preparation and IHC staining. Some DNA samples were a gift from Larry Donehower (Baylor College of Medicine, TX, USA). For mouse *K-Ras* and *N-Ras* amplification, primer sequences and cycling conditions were kindly provided by Lewis Chodosh and Robert Boxer (University of Pennsylvania School of Medicine).

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1741-7015/2/24/prepub