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Animal Model

Pathway Pathology

Histological Differences Between ErbB/Ras and Wnt Pathway Transgenic Mammary Tumors

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To study phenotype-genotype correlations, ErbB/Ras pathway tumors (transgenic for *ErbB2*, *c-Neu*, mutants of *c-Neu*, *polyomavirus middle T antigene* (*PyV-mT*), *Ras*, and bi-transgenic for *ErbB2/Neu* with *ErbB3* and with *progesterone receptor*) from four different institutions were histopathologically compared with Wnt pathway tumors [transgenes *Wnt1*, *Wnt10b*, dominant-negative *glycogen synthase kinase 3-β*, *β-Catenin*, and spontaneous mutants of *adenomatous polyposis coli gene* (*Apc*)]. ErbB/Ras pathway tumors tend to form solid nodules consisting of poorly differentiated cells with abundant cytoplasm. ErbB/Ras pathway tumors also have scanty stroma and lack myoepithelial or squamous differentiation. In contrast, Wnt pathway tumors exhibit myoepithelial, acinar, or glandular differentiation, and, frequently, combinations of these. Squamous metaplasia is frequent and may include transdifferentiation to epidermal and pilar structures. Most Wnt pathway tumors form caricatures of elongated, branched ductules, and have well-developed stroma, inflammatory infiltrates, and pushing margins. Tumors transgenic for interacting genes such as *protein kinase CK2α* (*casein kinase IIα*), and the fibroblast growth factors (Fgf) *Int2/Fgf3* or keratinocyte growth factor (*Kgf/Fgf7*) also have the Wnt pathway phenotype. Because the tumors from the ErbB/Ras and the Wnt pathway are so distinct and can be readily identified using routine hematoxylin and eosin sections, we suggest that pathway pathology is applicable in both basic and clinical cancer research. *(Am J Pathol 2002, 161:1087–1097)*

Genetically engineered mice (GEM) have been used extensively to model human breast cancer and to dissect the molecular pathways contributing to tumorigenesis. Most mammary tumors in GEM are different from spontaneous, virus-induced mammary tumors in mice.[1] As previously reported, many transgenes in GEM induce tumors with specific, signature histological phenotypes.[2] The initial observations were based on the cases collected in one laboratory at the Harvard Medical School. They demonstrated that the *Ras*, *Neu*, and *Myc* transgenes, promoted by the murine mammary tumor virus long terminal repeat (*MMTV-LTR*), produced signature tumors with small round cells, intermediate cells, and

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large cells, respectively. These observations suggested that phenotype predicts genotype.[2] Subsequent studies have shown that the signature phenotypes for these three genes are similar in different laboratories even with different constructs and different promoters.[3–5]

Since the initial studies, the University of California, Davis Mutant Mouse Pathology Archives have accumulated more than 3000 GEM mammary tumors that now include a sufficient variety of GEM to allow comparisons of tumors within specific signal transduction pathways. We have, therefore, undertaken a systematic study of the morphology of ErbB/Ras pathway tumors as compared with Wnt pathway tumors.

The *Wnt1* gene is one of the most commonly induced genes in mice after the insertional activation by MMTV (murine mammary tumor virus).[6] The *Wnt1* gene was first named "*Int1*", or MMTV integration site 1, until the activated gene was found to be the homologue of the Drosophila wingless gene.[7] The second gene commonly activated by MMTV insertion is known as *Int2* but has turned out to be a member of the Fgf family, *Fgf3*.[8] Although members of the Wnt and the Fgf families have been implicated in human cancer, neither has been frequently found in association with human breast cancer. A number of transgenic, mutant, and knockout mice have now been developed that involve the Wnt pathway.[9–12] Tumors in these GEM resemble the classical MMTV-induced tumors[13] suggesting that activation of the same gene by different mechanisms results in the same type of tumor. The MMTV-induced tumors have characteristic histological patterns that are not generally found in human breast cancer.[1]

Interestingly, *Wnt1* GEM infected with MMTV were found to have insertional activation of *Fgf8, Fgf4*, and *Fgf3* and GEM transgenic for *Fgf3* infected with MMTV were found to have activation of Wnt10b, suggesting cooperativity between Fgf and Wnt signaling.[6,9,14]

On the other hand, tumors arising in *ErbB2* GEM have a completely different histopathological pattern that does not resemble the MMTV-induced tumors[1] but rather do resemble some human tumors.[2] ErbB2 is a member of the epidermal growth factor receptor family and is amplified in ~25% of human breast cancer.[15] *Neu* is an activated rat homologue of *ErbB2*.[16] When either *c-ErbB2* or *Neu* is expressed behind the highly mammary selective MMTV-LTR promotor, a signature solid nodular tumor is generally produced.[3,4,17] Although some morphological differences may separate the Ras tumors from the Neu tumors, their phenotypes overlap to a significant degree.[2,18] Because polyoma virus middle T (PyV-mT) imitates ErbB2, it is considered a molecular surrogate for ErbB2.[19] PyV-mT protein also induces solid tumors.[19]

We now report that tumors involving other members of these two pathways in GEM share one or more morphological characteristics with the better known members of the Wnt or ErbB/Ras pathways. We describe here the morphological features shared by five members of the Wnt pathway and three cooperating genes as compared to those shared by six members of the ErbB2/Ras family. These studies extend the principle that phenotype predicts genotype to demonstrate that alterations in structure and function induced by genes can also be classified by the signal transduction pathway. Because these mouse mammary tumors have such different and easily identified morphologies, they belong to distinct taxonomic groups that are related to the pathways and we suggest the term "pathway pathology" to indicate the shared morphology within each pathway.

Materials and Methods

Mice

The samples used in this study came from murine tumors that were sent to us as a part of studies of oncogene tumorigenesis in transgenic mice initiated by our collaborators. All transgenes used here were under the control of an MMTV-LTR promotor except the PR− transgenics, created by using a binary system, as described previously.[20,21] All transgenic mice were bred in the FVB background strain. In addition, spontaneous *Min* mutants of the *adenomatous polyposis coli* (Apc^{Min}) gene in C57/BL/6J and (AKRx C57/BL/6J Min/+) F1 and N2 background were studied. Three of the six Apc^{Min} mice were treated with ethylnitrosourea, a chemical carcinogen.[22]

The animals were inspected for tumors at least once a week. Animals with tumors were necropsied between 1991 and 2001, and samples of tumors, adjacent mammary gland, and other tissues were fixed in neutral buffered 4% formalin or in Optimal Fix (American Master Tech Scientific, Inc., Lodi, CA), embedded in paraffin, cut into 4-μm sections, and stained with Mayer's hematoxylin and eosin (H&E). Animal data, gross description, slides, and, in most cases, paraffin blocks were stored at the University of California Davis Mutant Mouse Pathology Laboratory.

Tumors

ErbB/Ras Pathway

Representative mouse mammary tumors (n = 107) transgenic for the ErbB/Ras pathway were selected for this study. The transgenes were *ErbB2/Neu*,[3,23,24] *Neu* mutants *NDL1-4* and *NDL2-5*,[4] as well as transgenic crosses: *ErbB2* with *ErbB3* (Gillgrass and Muller, McMaster University, unpublished results), or with progesterone receptor α (n = 4) or β (n = 1) (G Shyamala, University of California, Berkeley, unpublished results). In addition, *PyV-mT*,[19,25,26] and *Ras*[2] tumors were examined.

Wnt Pathway

Tumors (n = 112) with one transgene or mutation activating the Wnt pathway were used. Bitransgenic tumors were not included because of the complexity of phenotypes in the Wnt pathway. The transgenes were *Wnt1*,[12] *Wnt10b*,[10] *protein kinase CK2*α (formerly *casein kinase IIa*),[11] β-*Catenin*,[12] dominant-negative mutant of *glycogen synthase kinase 3-b* (*dnGSK3*β) (D. C. Seldin,

unpublished results). In addition, *Apc^{Min}* mutants[27] were studied.

Int2 Pathway

Ninety tumors transgenic for *Int2/Fgf3*[28,29] or for *keratinocyte growth factor* (*Kgf/Fgf7*)[30] were studied.

The tumors were initially classified using the taxonomy recommended by the Annapolis Pathology Panel.[1] However, the remarkable phenotypes in the Wnt pathway tumors required the development of new taxonomic groups and terms (section results). Images were captured with ×10 and ×20 objectives using a Carl Zeiss (Thornwood, NY) Axiocam camera and were processed using Adobe Photoshop (Adobe Systems Incorporated) software.

Immunohistochemistry (IHC)

IHC was performed on 35 tumors transgenic for *ErbB2*, *Neu* mutants, and *PyV-mT*, and on 40 tumors transgenic for the Wnt pathway to assess myoepithelial differentiation, further on 5 tumors transgenic for the Wnt pathway to demonstrate ductular architecture, and on skin and 5 mammary pilar tumors to study pilar differentiation. Four-μm paraffin sections were placed onto Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, and cleared. IHC was performed after inhibition of endogenous peroxidase activity in a solution of 3% hydrogen peroxide (H_2O_2) in methanol and hydration in graded alcohol to distilled water. Before antibody incubations, antigen retrieval was performed by high temperature (microwave) incubation in 0.01 mol/L of citric acid buffer (pH 6.0) for 3 × 4 minutes. Slides were allowed to cool for 10 minutes in citric acid buffer then transferred to phosphate-buffered saline (pH 7.4) (2 × 5 minutes each). Ten percent normal horse serum (Vector Laboratories, Burlingame, CA) was applied to sections and incubated for 20 minutes in a humidified chamber at room temperature.

IHC for smooth muscle actin (SMA) was performed using a 1:1000 diluted mouse monoclonal primary antibody (Sigma, St. Louis, MO). IHC for hard (hair) keratin was performed using a 1:20 diluted cell culture supernatant with the mouse monoclonal primary antibody AE-13[31] (a kind gift from T.-T. Sun, New York University). The Animal Research Kit (DAKO, Carpinteria, CA) with peroxidase was used as amplification system according to the manufacturer's instructions.

To exclude SMA-positive myofibroblasts, all 12 questionable SMA-positive ErbB/Ras pathway tumors, two adenomyoepitheliomas, and 5 spindle cell tumors were stained for cytokeratin 14 (CK14). Staining for cytokeratin 8 (CK8) was performed to illustrate the ductular organization of Wnt pathway tumors. We used 1:200 (CK14) and 1:300 (CK8) diluted polyclonal sheep primary antibodies (Binding Site, San Diego, CA). Slides were covered with primary antibody solution and were incubated overnight at room temperature. The Vectastain ABC Elite Kit (Vector Laboratories) was used as amplification system according to the manufacturer's instructions. Slides were counterstained in Mayer's hematoxylin, dehydrated, cleared, and coverslipped. Negative control slides were run without primary antibody. Control slides known to be positive for each antibody were incorporated into each run.

Results

ErbB/Ras Pathway Tumors

ErbB2 and *Ras* transgenic mammary tumors have recognizable signature phenotypes as previously described.[1] *Ras* tumors consist of uniform cells with abundant eosinophilic cytoplasm and small ovoid nucleus with dense chromatin structure. *ErbB2/Neu* tumor cells are larger than *Ras* tumor cells, and have larger nuclei and paler but abundant cytoplasm (Figure 1E). *ErbB2/Neu* transgenic tumors are solid and nodular (Table 1). Solid *ErbB/Ras* pathway tumors (Figure 1, A and E) have characteristic concentric zones of cell populations: I, one to two peripheral layers of pallisading cells; II, several, more internal layers of larger cells with larger nuclei and more open chromatin structure (vesicular in the *ErbB2/Neu* tumors); and III, small, tightly packed central cells with smaller, elongated nuclei and less cytoplasm than in the other zones. Some tumors have central necrosis surrounded by bigger tumor cells than the zone III type cells. *PyV-mT* (Figure 1C) and *Ras* transgenic tumors have more variable phenotypes than *ErbB2* tumors (Table 1). Some solid *PyV-mT* tumors have minor components of glandular differentiation or cystic spaces. Although ErbB/Ras pathway tumors can have histological types other than solid (Table 1), they all contain solid components. With few exceptions, ErbB/Ras pathway tumors share common morphological characteristics.

The signature characteristics of the ErbB/Ras pathway tumors (Table 3) are: solid pattern (Table 1; Figure 1, A and E), scanty stroma (Figure 1; A, C, and E), invasive growth (Figure 1C), no myoepithelium (Figure 1E), and no squamous metaplasia (Figure 1; A, C, and E). The *PyV-mT* tumors tend to have more stroma than *Ras* and *ErbB2* tumors. Except for one tumor, ErbB/Ras pathway tumors do not have any evidence of milk or lipid secretion, even when the adjacent mammary gland is lactating. The connective tissue adjacent to the tumor has either no response or is edematous, but not fibrous (Figure 1E). Inflammatory infiltrates are limited to necrotic zones.

The ErbB2/Neu phenotype is consistently found in combinations of *ErbB2/c-Neu* transgenes with progesterone receptor or *ErbB3* transgenes.

Wnt Pathway Tumors

Mammary tumors induced by mutations in genes of the canonical Wnt pathway, or of the Wnt pathway interactors *CK2a*, *Int2* (*Fgf-3*), or *Kgf* (*Fgf-7*) (in the following referred to as Wnt pathway tumors) exhibit a variety of morphological patterns. Despite the variety of histological types (Table 2), these transgenic tumors have common histological characteristics, which are different from the ErbB/

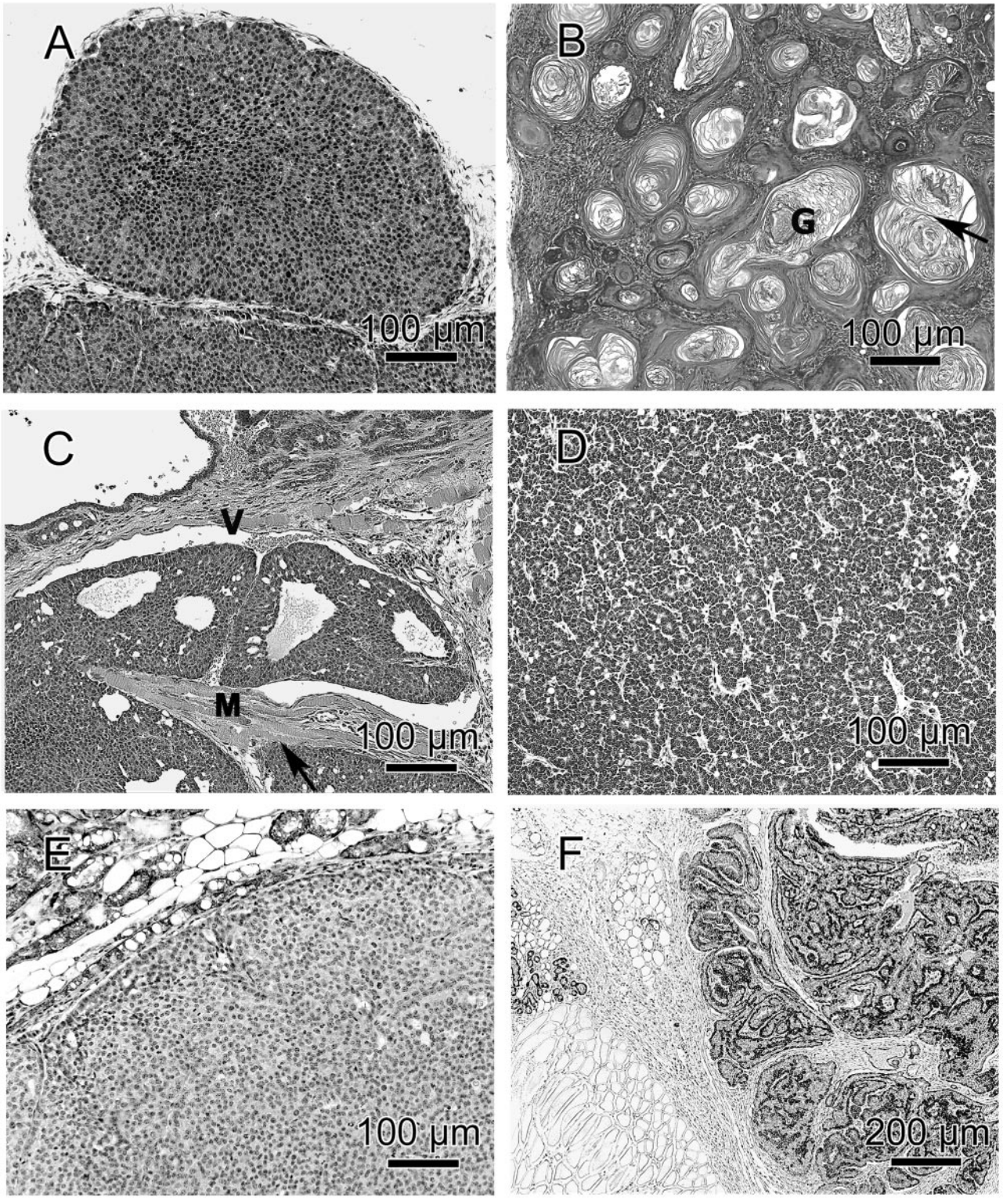

Figure 1. Representative histomorphology of ErbB/Ras pathway (**A**, **C**, **E**) and Wnt pathway (**B**, **D**, **F**) tumors. **A:** Solid nodular tumor with scanty stroma (transgene: *ErbB2/Neu* mutant *Ndl1-4*). **B:** Pilar mammary tumor with inflammatory infiltrates in well-developed stroma. Note central dilated neoplastic ducts filled with ghost cells (G) and laminar keratin, which forms concentric, and sometimes confluent, swirls (**arrow**). This tumor has both epidermal and hair-specific characteristics (mutated gene: *Apc*). **C:** Cystic-glandular tumor with solid components and scanty stroma invading into a dilated, thin-walled vessel (V) and into (**arrow**) the skeletal muscle (M) (transgene: *PyV-mT*). **D:** Acinar tumor with well-developed stroma (transgene: *β-Catenin*). **E:** SMA-negative solid nodular tumor with scanty stroma and no visible inflammatory infiltrate. Note that the myoepithelium in the adjacent dilated ductal and alveolar structures (**top left**) is SMA-positive (transgenes: *Neu* and *Progesterone receptor*). **F:** Stroma-rich type P tumor with inflammatory infiltrates. In both, tumor (**right**) and normal alveoli (**left**), the SMA-positive myoepithelium is located between luminal epithelium and stroma and forms a continuous line (transgene: *Wnt1*). **A–D:** H&E. **E** and **F:** Anti-SMA counterstained with hematoxylin. All photographs were taken with a ×10 or a ×20 objective, the exact scale is given in each picture.

Table 1. Histological Types of ErbB/Ras Pathway Transgenic Tumors

Transgene	Acinar	Glandular	Papillary	Solid	Adenosquamous	Pilar	Type P	Myoepithelial
ErbB2/Neu (n = 12)	0	0	1	11	0	0	0	0
activated *Neu* (n = 5)	0	0	0	5	0	0	0	0
Neu mutants (n = 25)	0	0	0	25	0	0	0	0
ErbB2 and *PR* (n = 5)	0	0	0	5	0	0	0	0
ErbB2 and *ErbB3* (n = 5)	0	0	0	5	0	0	0	0
PyV-mt (n = 49)	2	8	5	29	2	2	1	0
Ras (n = 6)	1	0	0	5	0	0	0	0
(n = 107)	3	8	6	85	2	2	1	0
(100%)	(3%)	(7%)	(6%)	(79%)	(2%)	(2%)	(1%)	(0%)

Ras pathway phenotype (Table 3). The key features of Wnt pathway tumors are branched ductular architecture (Figure 2, A and B), dense stroma with lymphocytic infiltrates (Figure 1, B and D, and Figure 2, A to D), and differentiation into acinar (Figure 1D), squamous (Figures 1B and 2D), and myoepithelial (Figure 1F and Figure 2, E and F) components. Each of these characteristics was found in more than 50% of the Wnt pathway tumors studied, and each Wnt pathway tumor had at least one of these characteristics.

Within the spectrum of Wnt tumor phenotypes are 1) better differentiated tumors exhibiting a ductular architecture (Figure 1, B and F, and Figure 2; A, B, and D); 2) less differentiated tumors without a ductular architecture (Figures 1D and 2E). Tumors with hints of ductular organization, but predominance of less differentiated components (Figure 2C), are classified with group 2.

The better-differentiated Wnt tumors have structures resembling elongated, branched ductules. The more peripheral portions of the tumor may have several patterns of terminal differentiation that may be classified according to the predominant pattern. The two types of well-differentiated tumors in this pathway are designated, here, as P-type and pilar tumors.

P-type tumors are characterized by ductules lined by single or multilayered epithelium and surrounded by dense stroma. The basal layer of myoepithelium is maintained (Figure 1F). The intraductal cells, especially in the periphery of the tumor, may differentiate into acinar, glandular, and papillary patterns or undergo minimal squamous metaplasia. However, the terminal ends of the ducts may also have masses of undifferentiated cells. Because this type of tumor phenotype has previously been described as "type P tumor" in conjunction with pregnancy-dependent, plaque-like tumors,[32] we chose to keep the term.

A second well-differentiated tumor also has caricatures of branched mammary ductules, but squamous metaplasia is present at the blind buds of the ductules, forming keratin-filled neoplastic ductules (abortive hair shafts) embedded in a fibrous stroma (Figures 1B and 2D). In most cases, ghost cells (Figures 1B and 2D), a typical component of pilomatricomas, are present. Pilar mammary tumors express hair-specific hard keratins as assessed by AE-13 antibody[31] (Figure 2D). The strong resemblance to hair structures and hair matrix-derived tumors has led us to designate these mammary tumors as "pilar tumors." The ductules of pilar tumors are filled with variable amounts of ghost (shadow) cells and keratin. These lumina are surrounded by basaloid cells (Figure 2D) and in some cases by an additional layer of myoepithelium. A subset of pilar tumors has been referred to earlier as "molluscoid tumor."[13] The periphery of this molluscoid subtype consists of abortive hair shafts. The centers of molluscoid tumors are filled with confluent swirls of laminar keratin (Figure 1B). These swirls allow the distinction from other keratin cysts. The swirls suggest that the keratin cysts of a pilar tumor are derived from individual neoplastic ductules that fuse together as they continue to produce keratin.

Pilar tumors are defined as mammary tumors composed of radially arranged hair shaft-like neoplastic ductules, or composed of a keratin cyst containing keratin swirls. In most cases, both components and ghost cells are present. The stroma of pilar tumors often has an intense inflammatory reaction. Pilar tumors may resemble squamous nodules, but they are larger and can metastasize. In other classifications, subsets of pilar tumors

Table 2. Histological Types of Wnt Pathway and *CK2α* and *Fgf* Transgenic Tumors

Transgene	Acinar	Glandular	Papillary	Solid	Adenosquamous	Pilar	Type P	Myoepithelial
Wnt1 (n = 7)	0	0	1	0	0	0	6	0
Wnt10b (n = 23)	7	4	1	1	0	1	9	0
CK2α (n = 29)	2	1	6	0	1	8	1	10
dnGSK-3β (n = 36)	1	6	8	0	2	11	1	7
Apc (n = 6)	0	0	0	0	0	6	0	0
β-Catenin (n = 11)	2	2	5	0	0	1	1	0
Int2 (n = 34)	6	6	7	0	5	7	1	2
Kgf (n = 56)	2	10	6	1	1	19	5	12
(n = 202)	20	29	34	2	9	53	24	31
(100%)	(10%)	(14%)	(17%)	(1%)	(4%)	(27%)	(12%)	(15%)

Table 3. Morphologic Criteria to Distinguish ErbB/Ras from Wnt Pathway Tumors

Criterion	ErbB/Ras pathway	Wnt pathway
Histological pattern	Solid	Branched ductules/ acinar component
Keratinization	Rarely present	Frequently present
Myoepithelium	Not present	Frequently present*
Stroma	Scanty	Dense
Inflammatory infiltrates	Present only in necrosis	Frequently present

*While myoepithelial spindle cell tumors and adenomyoepitheliomas represent only 15% of the Wnt pathway tumors, a basal myoepithelial layer is observed in more than 50% of the Wnt pathway tumors.

were referred to as adenosquamous carcinoma,[1] as squamous cell carcinoma[1] (the molluscoid subtype), or as intraductal squamous cell carcinoma.[33] These categories were not used to describe pilar tumors here because they include tumors other than pilar tumors. In Table 2, adenosquamous carcinoma[1] and squamous cell carcinoma[1] refer only to the nonpilar tumors of this category.

The less differentiated tumors found in the Wnt pathway are generally composed of one or more predominant histological patterns such as microacinar (Dunn type A) (Figure 1D), solid cords (Dunn type B), glandular, papillary (Figure 2C), squamous, or myoepithelial (Figure 2E). Tumors primarily composed of myoepithelial cells (Table 2) include adenomyoepitheliomas and spindle cell tumors (Figure 2E) and represent 15% of the Wnt pathway tumors. Some papillary tumors had components with pure micropapillary pattern. Some of the less differentiated tumors were adjacent to well-differentiated type P tumors.

The most characteristic histological pattern in the Wnt pathway tumors is squamous differentiation (Table 3). Squamous metaplasia may be extensive as in the pilar tumor or may be scattered as in other tumors. In addition, the majority of tumors also have acinar components. However, pure acinar tumors are rare. The majority of Wnt pathway tumors have some myoepithelial differentiation (Table 3) as confirmed by IHC for SMA (33 of 45 positive, Figures 1E and 2F) or for CK14 (7 of 7 positive, Figure 2E). The myoepithelium is either limited to a basal layer as in the better differentiated tumors (Figure 1F), or is the predominant population in the myoepithelial tumors (Figure 2E). Myoepithelium is also present in some pulmonary emboli and metastases (Figure 2F). Many Wnt pathway tumors have pushing tumor margins.

As might be expected from neoplasms with such complex phenotypes, Wnt pathway tumor cells have no one characteristic cytological feature. However, the cytoplasm of Wnt pathway tumor cells appears to be less abundant than in the ErbB/Ras pathway tumors with an inverted nuclear/cytoplasmic ratio. Thirty percent of the tumors had cytoplasmic lipid droplets corresponding to secretion, even though the adjacent mammary gland had involuted.

Discussion

We previously reported that GEM mammary tumors were frequently different from spontaneous MMTV-induced mammary tumors and many had recognizable signature phenotypes.[2] These observations led to the recognition that phenotype predicts genotype.[2] Currently, the University of California, Davis Mutant Mouse Pathology Laboratory archives include examples from more than 100 different GEM lines. Therefore, comparison of numerous genes involved in the same signal transduction pathways was possible. In the course of such studies, it became apparent that tumors from the members of the Wnt pathway could be distinguished from those affecting the ErbB/Ras pathway. Our observations expand the genotype predicts phenotype concept[2] to pathway pathology.

We previously described distinctive or signature phenotypes for *Erbb2/Neu* (Figure 1, A and E) and *Ras* transgenic tumors.[2] The *ErbB2* phenotype has been found to be remarkably consistent in a number of subtypes of the gene.[2,24,34] We found here that *ErbB2* and *Ras* tumors share morphological characteristics with the *PyV-mT* transgenic tumors (Table 3). Because *PyV-mT* acts as a surrogate for activated *ErbB2*, all three genes are members of the ErbB signaling pathway[35] (Figure 3). Some investigators have suggested that *ErbB2* tumors resemble human lobular carcinoma and originate from lobular hyperplasia.[36] Likewise, *PyV-mT* GEM have mammary glands with normal ducts and abortive lobules.[26] Both *PyV-mT* and *ErbB2* tumors completely lack myoepithelium (Table 3, Figure 1E). In fact, this myoepithelium is lost when *PyV-mT*-induced hyperplasia first becomes detectable.[26] Most ErbB pathway tumors consist of solid nests and cords. Tumor cell populations are frequently organized in concentric zones (see Results) and, in the predominant zone II, they have large nuclei with an open chromatin structure and abundant but undifferentiated cytoplasm.

As examples for Wnt pathway tumors, slides from four transgenic models of the Wnt pathway and from the spontaneous Apc^{Min} mutation (Figure 3) were studied. In addition, we analyzed tumors from a *CK2α* and two *Fgf* transgenic models. Our morphological study supports recently published data that CK2 is capable of promoting activation of the Wnt pathway through phosphorylation and stabilization of β-catenin and disheveled.[37] It is an important finding that tumors induced by *Kgf* and *Int2*, two members of the *Fgf* family, have the same phenotype as Wnt pathway tumors, referred to as Wnt pathway phenotype. We hypothesize that these two genes not only cooperate with the Wnt pathway[6,9,14] but may also activate this pathway. This hypothesis is supported by the observation that MMTV infection activates *Fgf* family members in *Wnt* GEM, and activates *Wnt* family members in *Fgf* GEM.[9,14]

Several features were identified that were characteristic for the Wnt pathway tumors, and were rarely, if ever, observed in the ErbB/Ras pathway tumors (Table 3; Figure 1, A to E). These include 1) branched ductal architecture; 2) differentiation into squamous, acinar or glandular, solid, and/or myoepithelial components; and 3)

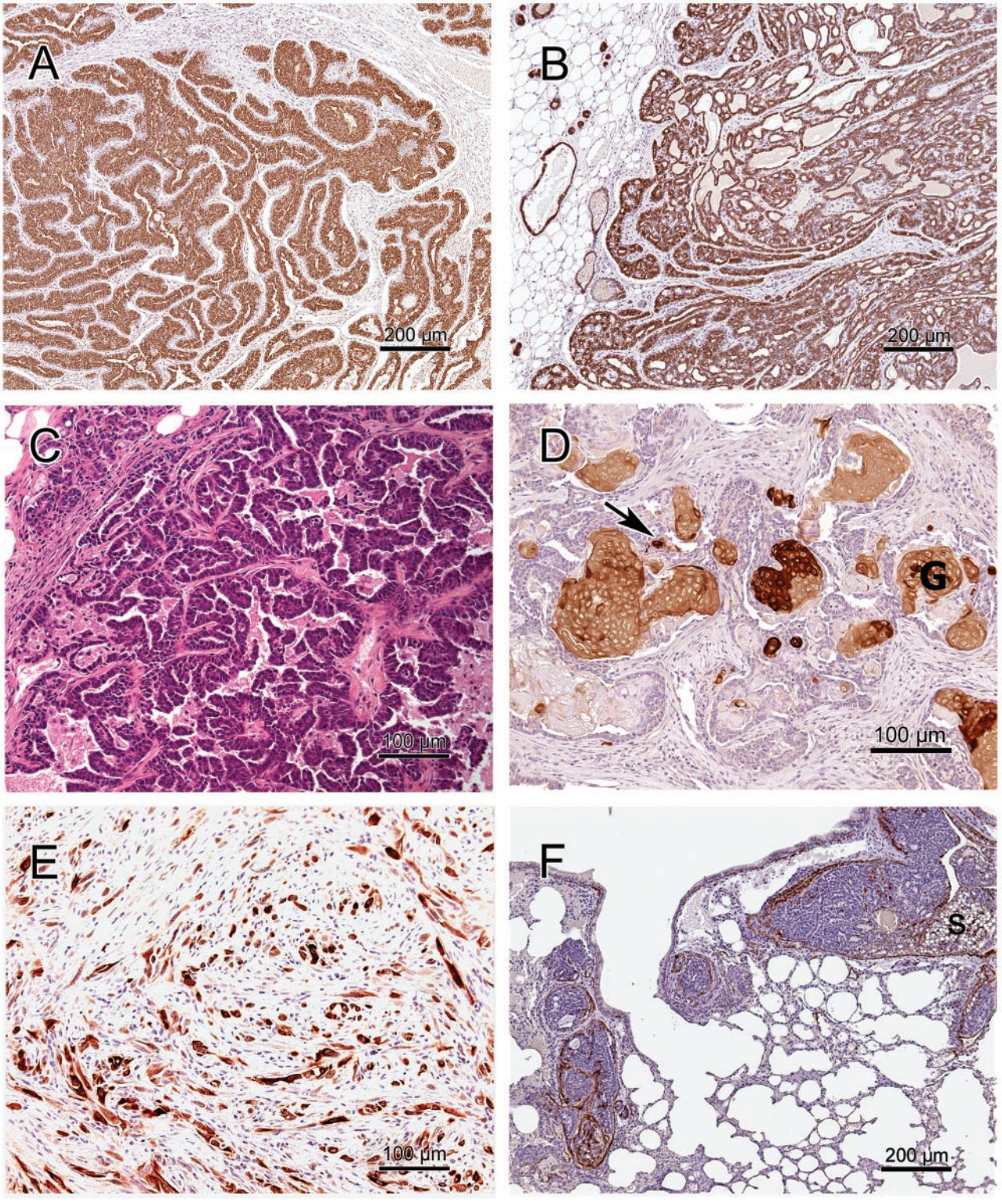

Figure 2. Characteristics of Wnt pathway tumors: Ductular architecture (**A**, **B**) with glandular (**B**), papillary (**C**), pilar (**D**), and myoepithelial differentiation (**E**, **F**), and dense stroma with lymphocytic infiltrates (**A–D**). **A:** Type P tumor. Note the well-differentiated branching neoplastic ductules (transgene: *Wnt10b*). **B:** Type P tumor with glandular differentiation (transgene: *Wnt1*). **C:** Papillary tumor with micropapillary components. The ductular architecture is less prominent than in type P tumors (transgene: *CK2a*). **D:** Pilar tumor with squamous metaplasia and ghost cells (G) embedded in fibrous stroma with moderate inflammatory infiltrates. Staining with antibody AE-13 shows that expression of hair keratin in viable cells is limited to few single cells (**arrow**) (transgene: *Int2*). **E:** Myoepithelial differentiation in a spindle cell tumor (transgene: *CK2a*). **F:** Lung metastasis of a glandular mammary tumor with secretory activity (S) and maintained myoepithelium. In mice, maintained myoepithelium does not exclude tumor emboli or metastasis (transgene: *Kgf*). All photographs were taken with a ×10 or a ×20 objective, the exact scale is given in each picture. **A** and **B:** Anti-cytokeratin-8; **C:** H&E; **D:** AE13 (anti-hair-keratin); **E:** anti-cytokeratin-14; **F:** anti-SMA counterstained with hematoxylin.

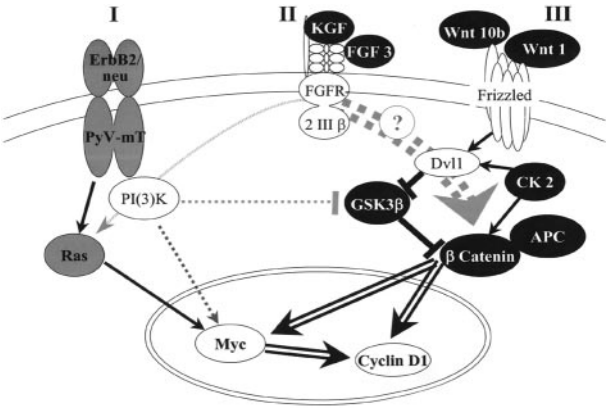

Figure 3. ErbB/Ras pathway and Wnt pathway. The morphological phenotypes of transgenic tumors reflect the signaling pathway activated by the transgene. The color of the studied transgenes indicates the observed phenotype: gray for ErbB2/Ras pathway-like, and black for Wnt pathway-like. **Black links** between genes indicate activation or inhibition. **Gray dotted links** show interpathway links, and **open arrows** symbolize transcriptional activation. **I:** ErbB2/neu and its intracytoplasmic substitute PyV-mT activate the phosphoinositol-(3) kinase [PI(3)K][19] and the Ras pathways,[35] leading to accumulation of Myc and cyclin D1 in the nucleus.[53,63,64] **II:** Fibroblast growth factor 3 (FGF3) [synonym, MMTV integration site 2 gene (Int2)] and keratinocyte growth factor (KGF) [synonym, fibroblast growth factor 7 (FGF7)] both bind to the same receptor, fibroblast growth factor receptor 2 III β (FGFR 2 III β).[65] The intracellular signal transduction varies depending on organ, tissue maturity, and species.[66–68] Our results suggest an activation of the Wnt pathway in *FGF* transgenic mouse mammary tumors. **III:** Wnt1 or Wnt10b bind to Frizzled,[69] and activate the mouse dishevelled homolog 1 (Dvl1). Protein kinase CK2 (CK2) also phosphorylates and stabilizes β-catenin and Dvl1.[37] Dvl1 inhibits the activity of glycogen synthase kinase-3β (GSK3β), leading to an accumulation of β-catenin.[70] The degradation of β-catenin depends on complex formation with several proteins including the adenomatous polyposis of the colon gene product (APC). If β-catenin accumulates, it translocates into the nucleus and induces the transcription of target genes, including *Myc* and *Cyclin D1*.[37,54]

well-developed stroma and host response. Because these patterns have not been emphasized or grouped in previous publications, they are discussed in more detail below.

Branched Ductal Architecture

The characteristic feature of well-differentiated Wnt pathway tumors is the organization of the tissue around irregularly branched, elongated ductules. Since this phenomenon has previously been described,[13] we used the old term "type P tumor" (see Results and Figure 1F and Figure 2, A and B). However, the branching morphogenesis could also be identified in association with less differentiated tumors with predominant glandular, papillary, or pilar differentiation. The fact that some poorly differentiated tumors were in direct continuity with well-differentiated tumors suggests that the less differentiated tumors arose from subpopulations of cells within the originating tumor.

The Wnt pathway is critical for tissue and organ differentiation.[38] Different components influence cell fate decisions. The ductal dysmorphogenesis that seems to be so characteristic of the Wnt pathway tumors is in contrast to the lobular dysmorphogenesis observed in the ErbB pathway. However, the reader is to be reminded that the classical consequence of MMTV infection is the hyperplastic alveolar nodule, which is clearly the result of lobuloalveolar differentiation. In our experience, the MMTV-induced hyperplastic alveolar nodule is associated with *Wnt1* activation whereas the type P tumor is primarily associated with *Int-2*.[39]

Terminal Differentiation

The aberrant ductules described above terminate in masses of cells that appear to differentiate along several different pathways. As suggested, the same tumor may have foci that have differentiated along different pathways.

Squamous metaplasia and keratinization was found in, but not limited to, pilar tumors (see Results). The frequent presence of squamous metaplasia in the transgenic Wnt pathway tumors may be related to the dysregulation of *β-Catenin*[40] (Figure 3). The spontaneous mouse mammary tumors, that are primarily associated with *Wnt1* activation,[39] seldom have squamous metaplasia.[13] As observed here, ErbB/Ras pathway tumors rarely have squamous metaplasia. Squamous differentiation was less frequent in *Wnt1* and *Wnt10b* GEM than in GEM with mutations in genes downstream from Wnt (Table 2 and Figure 3). It is possible that the Wnt glycoproteins activate noncanonical pathways[41] that promote acinar or glandular differentiation and prevent keratinization.

Pilar tumors (see Results and Figure 1B), a distinct histological type with squamous differentiation, represent the most common histological type in the Wnt pathway tumors (Table 3). Pilar tumors may have ghost cells (Figures 1B and 2D), neoplastic ductules resembling abortive hair shafts, or squamous cysts that appear to be derived from confluent ductules (Figure 2B) characterized by swirls of laminar keratin on the cross-section. Pilar tumors are an unusual phenotype for the mammary gland, but closely resemble trichoepitheliomas or pilomatricomas, skin or hair matrix-derived tumors (Figure 2, C and D).

Accumulation of *β-Catenin* induces *de novo* hair morphogenesis and trichoepitheliomas in the skin.[42–44] In the mammary gland, a skin appendix, the Wnt/*β-Catenin* pathway promotes both epidermal transdifferentiation[71] and hair-specific features (Table 2, Figures 1B and 2D).

All mammary tumors in mice bearing a mutation of the Apc^{Min} gene were pilar (Table 2). Mammary and intestinal tumors in Apc^{Min} mice were extensively studied in various backgrounds regarding spontaneous and chemical carcinogenesis.[22] Treatment with chemical carcinogens in mice frequently induces squamous mammary tumors. However, the pilar phenotype was also observed in spontaneous Apc^{Min} tumors and in various backgrounds. Interestingly, chemically induced adenocarcinomas with squamous metaplasia in nontransgenic mice have *Ras* mutations in 20%.[45,46] However, the six *Ras* transgenic tumors included in our study had no keratinization.

Squamous metaplasia of the lactiferous ducts, in humans, is related to smoking (just another chemical carcinogen), and is often associated with periductal inflammatory infiltrates[47] and with inflammatory pseudocapsules of silicon prosthesis implants.[48] We observed a similar association of inflammatory infiltrates and squamous metaplasia

in the murine Wnt pathway tumors (Table 3 and Figure 1B). Squamous metaplasia is also found in 4% of human breast carcinomas.[48]

Microacinar and glandular differentiation and secretory activity were frequent in the Wnt pathway tumors (Tables 2 and 3; Figure 1, D and F; and Figure 2, B and F). Although only a few tumors were pure classical MMTV-induced microacinar type A tumors as described by Dunn, minor microacinar components were characteristic for many Wnt pathway tumors. This supports the concept of pathway pathology (Table 3, Figure 1D). Although some ErbB/Ras pathway tumors are papillary, they rarely have significant glandular differentiation.

Solid cords of cells with peripheral palisades of SMA-positive cells are characteristic of the classical type B tumor.[13] Solid tumors are rare in transgenic Wnt pathway tumors (Table 2). However, the ends of some of the terminal ducts contain solid masses of undifferentiated cells that do not express the epithelial marker CK8.

Myoepithelial differentiation is also characteristic of Wnt pathway tumors (Figure 1F; Figure 2, E and F; Table 3). In contrast, the loss of myoepithelium marks *PyV-mT* and *ErbB2* atypia and neoplasia[26] and most human breast cancers.[49,50] The presence of myoepithelium in the Wnt pathway tumors may be significant in that the myoepithelium is considered a natural tumor suppressor.[51,52] The myoepithelium appears as a distinctive basal layer in many Wnt pathway tumors. However, the Wnt pathway induced spindle cell tumors also proved to be myoepithelial (Figure 2E and Table 2). Because spindle cell tumors are concentrated in the *Kgf*, *CK2a*, and *dnGSK3b* genotypes, this variation of phenotype may be because of additional pathways activated by these genes (Figure 3).

Stroma

In contrast to the ErbB/Ras pathway tumors, the stroma of most Wnt pathway tumors was well developed and contained inflammatory infiltrates, predominantly lymphocytes (Table 3; Figure 1, B and F; Figure 2, A to D). The host-tumor interface had a pushing margin in many Wnt pathway tumors (Figure 1F) in contrast to the more invasive growth of the ErbB/Ras pathway tumors (Figure 1C).[3,25]

Two target genes activated by both pathways are frequently amplified in human breast cancer: *myc* and *cyclin D1* (Figure 3). The dependence of Wnt- and ErbB2-induced tumorigenesis on cyclin D1 has recently been documented by Yu and co-workers.[53] The phenotype of tumors induced by these target genes will be discussed elsewhere (A. Rosner, R. D. Cardiff, and J. P. Gregg, unpublished data).

Conclusions

We compared the histology of ErbB/Ras and Wnt pathway transgenic mammary tumors. Wnt pathway tumors frequently show combinations of acinar, glandular, myoepithelial, or pilar differentiation. Despite the complexity of differentiation patterns in the Wnt pathway tumors, the morphological criteria given in Table 3 distinguish Wnt pathway tumors from ErbB/Ras pathway tumors. We should emphasize that the lesions described here are only signature lesions that are closely related to the genotype. As is recorded in the tables presented here and elsewhere, any given GEM genotype can give rise to a range of tumor phenotypes. However, the tumors described here are so characteristic of the pathway that, when identified in histological sections, they are pathognomonic of the pathway and, thus, should be placed in a separate part of the taxonomic nomenclature related to the pathway. Hence, pathway pathology should result in a specific taxonomic category for mouse tumors.

Pathway pathology should have applications in both basic and clinical research. Histomorphological criteria can help to identify unexpected activated signaling pathways in transgenic tumors. For example, the relationship between the morphology of *Fgf* and Wnt pathway tumors implies the utilization of the same molecular processes. Our studies also suggest that a subset of keratinizing or myoepithelial human breast tumors might have activation of the Wnt pathway.

In human disease, the diagnosis of the oncogenic genetic aberrations is increasingly important for predicting prognostic and therapeutic strategies. Overexpression of *ErbB2* is a well-established example of molecular profiling. The subgroup of breast cancer patients with tumors expressing *ErbB2* benefits from treatment with a humanized anti-*Her2/Neu* monoclonal antibody (Herceptin).[55,56] The need for genetic profiling is growing because of the rapid development of strategies based on gene therapy.[57–59]

Studies in human breast cancer, based on standard histological classifications, have found several correlations between the tumor phenotype and genotype. Ductal carcinoma *in situ* often has an amplification or overexpression of *ErbB2*.[15] Mutations of the *BRCA1* gene are especially frequent in medullary carcinoma.[60,61] Lobular carcinomas are associated with deletion of E-cadherin.[62]

Our research now suggests that these concepts need to be expanded to include entire pathways. The correlations should become even stronger as additional criteria are recognized and as pathways and phenotypes are compared with gene expression signatures. Furthermore, as targeted molecular therapeutics become more widely available in the future, these correlations will have increasing clinical importance.

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Note Added in Proof

Since the submission of this paper, an article has appeared that documents hierarchical clustering of tumor types at the level of gene expression. (Desai KV, Xiao N, Wang W, Gangi L, Greene J, Powell JI, Dickson R, Furth P, Hunter K, Kucherlapati R, Simon R, Liu ET, Green JE. Initiating oncogenic event determines gene-expression patterns of human breast cancer models. Proc Natl Acad Sci USA, 2002 99:6967–6972.)

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