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Transgenic Oncogene Mice

Tumor Phenotype Predicts Genotype

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The hypothesis that oncogenes influence tumor phenotype was tested by examining slides from 607 mammary tumors from 407 transgenic mice bearing the* ras, myc, *and/or* neu *oncogenes. Most tumors (91%) had patterns (phenotypes) that could not be classified by Dunn's standard nomenclature. The nonstandard tumors were described as eosinophilic small cell (SC), basophilic large cell (LC), or pale intermediate cell (IC). The SC tumor was associated with* ras, *the LC was associated with* myc, *and the IC was associated with* neu, *with specificities more than .90 and sensitivities ranging from .99 to .48. Thus, the tumor phenotype could be used to predict which oncogene was present in the animal. The presence of* myc *in combination with either* ras *or* neu *resulted in the predominance of LC tumors and accelerated tumorigenesis. The combination of* ras *and* neu *resulted in a decreased tumor incidence. Thus, knowledge of the oncogenes that were present could be used to predict the natural history of the disease. (Am J Pathol 1991, 139:495–501)

Oncogenes have received increasing recognition as important prognostic factors in clinical oncology.[1,2] They have been associated with prognosis in certain types of lymphomas and leukemias.[3] However, with few exceptions, activated oncogenes have not been associated with specific histologic patterns in epithelial tumors.[2,4–7] This may be attributed, in part, to the few attempts to correlate pathologic and molecular analysis.

We reviewed a series of histopathologic slides of mammary tumors from transgenic mice. The results of our study lend credence to the hypothesis that specific oncogenes may exert a sufficient influence on the tumor phenotype so that the histologic pattern can be used to predict the oncogenes activated in tumors of unknown molecular origin.[4] This observation raises the possibility that activating oncogenes may be predicted by the histologic features of human carcinomas.

In the inquiry reported later, we describe the first systematic comparative investigation of murine mammary tumor phenotype and genotype in transgenic mice. When placed behind a murine mammary tumor virus promoter, the oncogenes *ras*, *myc*, and *neu*, which are also implicated in human breast cancer, increase the incidence of mammary tumors.[6–11] We describe three histologic patterns of tumors that are highly specific for the *ras*, *myc*, and *neu* oncogenes. When two or more oncogenes are present, the tumor type associated with the *myc* oncogene appears to dominate the other two genes. Data is also presented that suggests that the tumor genotype influences the natural history of tumorigenesis. Although these observations are limited to animals, they have important implications for carcinogenesis in humans. Our studies should encourage the student of pathology to search for similar relationships in human epithelial tumors.

Materials and Methods

Mice

The mice that were used in this study were a part of a study of oncogene tumorigenesis in transgenic mice. All oncogene constructs were driven by a MuMTV LTR promotor placed in front of the *neu*, *ras*, or *myc* oncogenes.[9] The TG.SH and TG.SI strains were produced in 1986 by injection of FVB mice with a *ras* construct.[10] The TG.M

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and TG.K strains were produced using a *myc* construct.[8] The TG.NK and TG.NF strains were produced with a *neu* construct.[11] Bigenic animals were produced by crossing either TG.M or TG.K with TG.SH mice. Potentially trigenic TG.NKMSH animals were produced by crossing heterozygous TG.NK mice with heterozygous TG.MSH, which created eight possible genotypes. The genotype of each animal was established by extracting portions of the tail and performing Southern blots as previously described.[8–11]

Pathology

The animals were inspected for tumors at least once a week by one of the investigators. Tumors were recorded on the Harvard Medical School Department of Genetics VAX computer (Digital Corp, Maynard, MA) using the VAX Editor Program. Animals with tumors were autopsied, and samples of tumors and representative samples of other organs were fixed in formalin, processed, and stained with eosin and hematoxylin. All of the tissue was collected between 1986 and 1988. The slides were stored in the archives of the Department of Genetics, Harvard Medical School, until the current study.

A total of 607 tumors from 407 mice were analyzed by one of us (RDC). The pathologist examined each slide and recorded his impression of the histologic pattern of each tumor. Since the slides had been previously labelled, no attempt was made to perform a blinded study. However, the examiner did not decode the genotype of the animals until the study was completed. The pathologic interpretations were limited to those slides that were still on file. Pathologic diagnoses, which were recorded but could not be verified by review of the actual slide, were eliminated from consideration.

Data Management

The animal data were stored on a computer in the Harvard Medical School Department of Genetics. The pathology, genotype, and tumor latency data were stored, and calculations were done using the Microsoft Excel program. All animals in the databank were included in the initial studies and were used to calculate tumor incidence. The tumor incidence was calculated by taking the total number of animals in the transgenic group that had either developed a tumor or had attained the age equal to or greater than the mean tumor latent period for that group. However, animals with ambiguous or incomplete archival data were removed from the calculations that involved the histologic slides. Thus, the number of animals in the slide study did not always correspond to the larger data analyses that were based on the material in the computer databank.

The statistical analysis was performed by Mr. Larry Carr with the DEC VAX/VMS version V5.3 at the University of California, Davis Computing Service. SAS programs were used for Tukey's studentized range (HSC) test for variable: A, and Boniferroni (Dunn) T test for variable: A.

Results

Histology

More than 95% of the mammary tumors occurring spontaneously in laboratory mice that were infected with the mouse mammary tumor virus can be categorized using the Dunn classification.[12] In contrast, only 9% of the tumors from the transgenic mice could be placed into standard categories as described by Dunn, referred to below as "Dunn tumors" (DT).

The majority of mammary tumors that were found in the transgenic mice exhibited histologic patterns that, in our experience, are rare or have not been previously described (Table 1). The bulk of these transgenic tumors could be divided into three general categories: 1) eosinophilic, papillary adenosquamous small cell (SC), 2) basophilic, glandular large cell (LC), and 3) pale nodular intermediate cell (IC).

The SC tumors (Figure 1A) had uniform, small round-to-oval nuclei with a diffuse chromatin pattern and a low mitotic rate. The cytoplasm was variable but tended to be sparse and was distinctly eosinophilic. The cells tended to grow in vaguely nodular multilayered sheets with the cells oriented perpendicular around the blood vessels that emulating papillary fronds. Some of these tumors also produced layers of cells and glandular patterns giving a adenosquamous differentiation.

The LC cells (Figure 1B) had larger, more pleomorphic nuclei with vesicular chromatin patterns and prominent nucleoli. The mitotic rate was high. The cytoplasm, although generally sparse, was distinctly basophilic. The cells characteristically grow in an ill-defined glandular pattern or in more diffuse sheets.

Table 1. *Monogenic Animals*

Genes	No. tu	No. mice	Mammary tumor types			
			SC	LC	IC	DUNN
ras	193	145	145	1	0	47
myc	11	9	1	7	0	3
neu	61	39	18	0	31	12
Total	265	193	164	8	31	62

SC, small cell; LC, large cell; IC, intermediate cell; DUNN, tumor types classified by Dunn.[12]

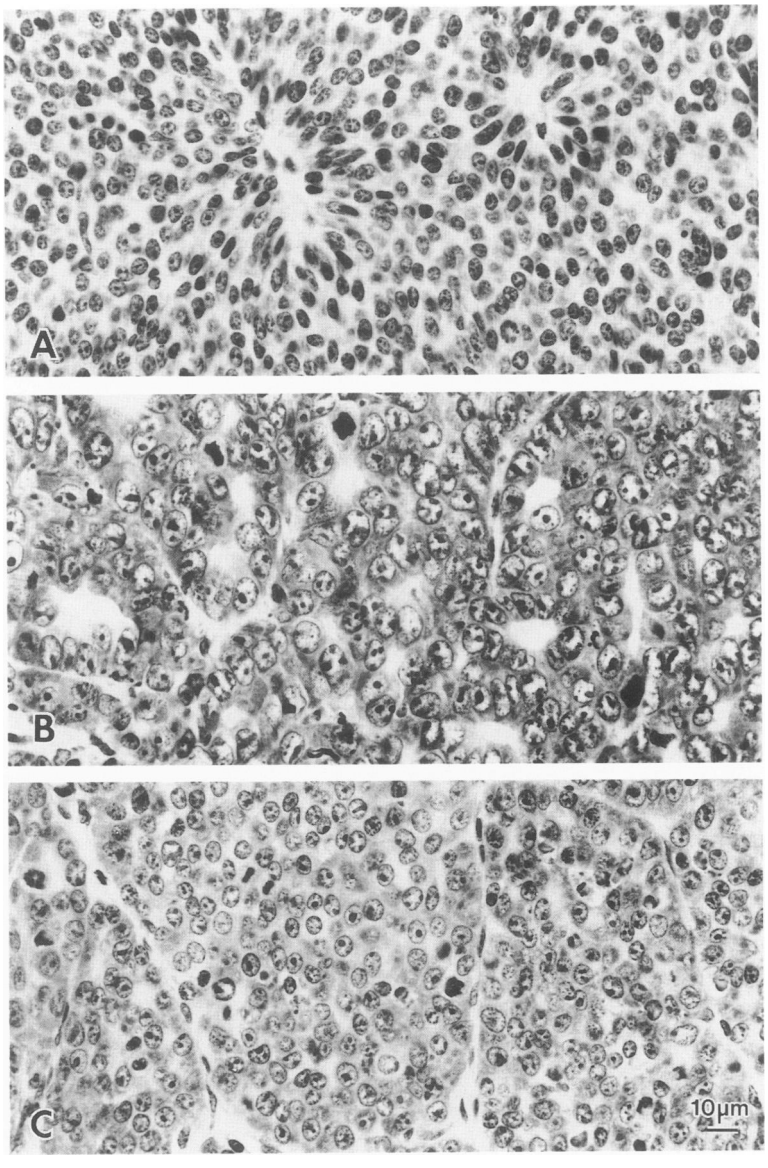

Figure 1. **A**: *High-magnification photomicrograph of eosinophilic, small cell tumor that is frequently associated with the* ras *oncogene. Note the round-to-oval small nuclei and pale cytoplasm. The cells are oriented perpendicular around the blood vessels.* **B**: *A basophilic, large cell tumor that is frequently associated with the* myc *oncogene photographed at the same magnification as* **(A)** *and* **(C)**. *Note the pleomorphic nuclei with open chromatin patterns and prominent nuclei. The cells have dark-staining cytoplasm and are organized as psuedoacini.* **C**: *High-magnification photomicrograph that shows the pale-staining nodular intermediate cell tumor that is associated with the* neu *oncogene. Note the more uniform nuclei than the SC tumor and the a more open chromatin pattern than the LC tumor. The cells have a pale pink-staining cytoplasm and are organized as nodules. The bar in the lower-right corner indicates 10* µm.

The IC cell (Figure 1C) had a round nucleus, which was intermediate in size, with a slightly more open chromatin pattern than the small cell tumors. The cells had an almost clear, pink cytoplasm. These tumors tended to be nodular, expansile masses that frequently had a peripheral palisade of nuclei or were organized around ill-defined ductular structures.

Of the three types, the LC tumors were the easiest to separate from the other two types. The SC and IC tumors were more difficult to separate. They seemed to represent overlapping groups with the SC tumors at one end and the IC tumors at the other. Some of these tumors had a mixture of cell types or histologic patterns. The tumors were placed into one of the four categories on the basis of the predominant cell type.

Correlation with Monogenic Genotype

Tumors from transgenic animals that bore a single oncogene were classified according to the criteria described earlier. As seen in Table 1, 145/193 (75%) of the tumors from animals with the *ras* oncogene were classified as SC tumors. Only 1 of 193 tumors had the characteristics of the LC tumors. Further, only 47/193 (24%) of the tumors were Dunn type (DT).

Although a limited number of *myc* transgenics were available for examination, the majority (7/11) exhibited the LC phenotype. The IC tumors were limited to the *neu*-bearing transgenic animals (Table 1). As indicated, 31/61 (50%) of the tumors in these animals were classified as IC tumors and 18/61 (30%) were indistinguishable from the

SC tumor. Most significant, none of the tumors in the *neu* transgenic animals had the LC phenotype.

Correlation with Bigenic and Trigenic Genotype

A more rigorous study was carried out in bigenic animals that were F1 hybrids between heterozygous *ras*- and *myc*-transgenic parents. Because the animals that were used were all heterozygous, four different genotypes were possible. In these studies, the slides were read, and the tumors were classified before the genotypes were known. Since the pathologist did not have access to the genotype of the animal, this portion of our study represented a blinded control, which tested the validity of our criteria.

Fifty percent of the tumors that were found in the bigenic animals were type LC (Table 2). When the tumor phenotype was correlated with the animal genotype, 41/44 (93%) of the LC tumors were found in animals that had one *myc* allele. Without the detailed expression data, one can only speculate that the other three tumors had *myc* activated through another mechanism. In contrast, 100% of the SC tumors occurred in animals with a *ras* allele. Nineteen of the 25 (76%) SC tumors were found in the animals with only the *ras* allele (Table 2). The other six SC tumors were in bigenic animals that had both the *ras* and the *myc* alleles. None of the 81 wild-type animals that were observed for 180 or more days had mammary tumors develop.

A set of 59 trigenic animals was studied as an even more complex genetic interaction (Table 3). The trigenic animals were a F1 hybrid between heterozygous *myc*/*ras*- and *neu*-bearing transgenics. Because the animals that were used were all heterozygous, eight different genotypes were possible.

As in the bigenic mice, more than half 40/75 (53%) of the tumors had the LC phenotype. The SC phenotype was second most common with 22/75 (29%) of the tumors. Only 2 of the 75 tumors could clearly be classified as type IC.

When the genotype of the trigenic animals was matched with the tumor phenotype, 40/41 (98%) of the LC tumors were associated with at least one *myc* allele. The presence of the *myc* allele, even in the presence of the other two alleles, was associated with a predominance 39/57 (67%) of LC tumors (Table 3). The majority of tumors that were classified as DT were in the *myc*-bearing animals. In fact, 49/57 (86%) of all tumors that were found in *myc*-bearing mice were either DT or LC types.

All (22/22) of the SC tumors were found in animals with the *ras* or the *neu* allele. However, 10 tumors were in animals with *neu*/+/+ genotypes. Only six of the SC tumors were in mice with the *ras* oncogene in the absence of the *myc* gene. None of the 64 wild-type animals developed mammary tumors.

These impressions were supported by application of the standard sensitivity, specificity, and predictive value tests to the data. In Table 4, these values were computed on the basis of individual animals. In compiling the data, it was assumed that *myc* dominated *ras* in bigenic or trigenic mice and that *ras* dominated *neu*. Therefore, animals with the *myc* oncogene and LC phenotype were not scored as false or true negatives if they had either of the other two alleles. Further, when tumors of different phenotypes appeared in the same multigenic animal, each tumor was scored in relation to the genotype. The data that were computed in this manner showed that the phenotypes were highly specific but varied in sensitivity and predictive value (Table 5). This suggests that the presence of the given phenotype was important but that the absence of that phenotype was less predictive.

Relation of Phenotype/Genotype with Tumor Kinetics

The tumor development data for each major strain and cross that was used are found in Table 5. Because many

Table 2. *Bigenic Animals*

Genes	No. tu	No. mice	Mammary tumor types SC	LC	IC	DUNN
myc/ras	62	45	6	40	0	16
myc	1	1	0	1	0	0
ras	25	20	19	3	0	3
Total	88	66	25	44	0	19

SC, small cell; LC, large cell; IC, intermediate cell; DUNN, tumor types classified by Dunn.[12]

Table 3. *Trigenic Animals TG.NKMSH*

Genes	No. tu	No. mice	Mammary tumor types SC	LC	IC	DUNN
myc/ras/neu	24	17	3	18	2	1
myc/neu	25	19	1	16	0	8
myc/ras	7	5	2	5	0	0
myc	1	1	0	0	0	1
neu	10	10	10	0	0	0
neu/ras	6	5	4	1	0	1
ras	2	2	2	0	0	0
Totals	75	59	22	40	2	11

SC, small cell; LC, large cell; IC, intermediate cell; DUNN, tumor types classified by Dunn.[12]

Table 4. *Correlation of Genotype with Tumor Phenotype*

	myc	*ras*	*neu*
Sensitivity	0.75	0.87	0.48
Specificity	0.91	0.98	0.99
+ Predictive value	0.81	0.99	0.91
− Predictive value	0.88	0.80	0.88

Table 5. *Tumor Incidence*

Breed	Genes	No. mice	No. tu	% Tu	Mean tumor latent period (SD)
TG.SH	*ras*	181	102	56	161(93)
TG.M	*myc*	NA	32	NA	277(125)
TG.NK	*neu*	70	35	50	202(75)
TG.NF	*neu*	67	64	96	97(14)
TG.MSH	*myc/ras*	64	45	70	113(50)
TG.MSH	*myc*	56	6	11	231(84)
TG.MSH	*ras*	72	22	31	138(100)
TG.MSH	wild type	81	0	0	—
TG.NKMSH	*myc/ras/neu*	17	17	100	72(31)
TG.NKMSH	*myc/ras*	10	10	100	89(28)
TG.MKMSH	*myc/neu*	22	20	91	141(57)
TG.NKMSH	*neu/ras*	16	6	38	180(155)
TG.NKMSH	*myc*	10	2	20	299(NA)
TG.NKMSH	*ras*	6	4	67	263(14)
TG.NKMSH	*neu*	28	20	73	228(51)
TG.NKMSH	wild type	64	0	0	—

NA, data not available.

of the founder animals were outbred, the significance of the difference in mammary tumor development in the monogenic animals is difficult to assess. However, the bigenic and trigenic crosses provide the opportunity to compare the oncogenes on a more homogeneous genetic background. The combination of all three oncogenes led to 100% tumors with the earliest mean latent period (Table 5, Figure 2). Bigenic crosses with *myc* + *ras* or *myc* + *neu* had a higher incidence in a shorter mean latent period than most of the monogenic animals. The exception to this rule was the TG.NKMSH-derived *neu* + *ras* bigenic, which had a low tumor incidence (38%), suggesting a different type of interaction between these two oncogenes.

The mean latent periods were, for the most part, not statistically different. However, some exceptions are notable. For example, the latent periods of the *ras*-bearing TG.SH and the *myc*-bearing TG.MSH transgenics were different from the *neu*-bearing TG.NK mice at the .95 confidence limit using the Tukey's studentized range test (Table 5). The latent period was also affected by the transgenic strain. For example, the transgene *neu* was associated with a latency of 96 days in the TG.NF transgenic strain, whereas the TG.NK strain had a mean latency of 202 days (Table 5) (confidence limits >.95).

Trigenic animals carrying all three oncogenes had the shortest mean latent period of 72 days and had a cumulative tumor total with a sharp descent (Figure 2). The bigenic combinations of *myc/ras* and *myc/neu* from the TG.NK × TG.MSH cross had similar slopes, whereas the other genotypes had longer latency periods and shallower slopes. The mean latent period of trigenic NK.MSH mice that had all three alleles (*myc/ras/neu*) was also significantly different at the .99 limit from the monogenic animals from the same crosses bearing the individual oncogenes.

Discussion

The *ras, myc,* and *neu* oncogenes have been implicated in the development of human breast cancer.[1–7] They typically are amplified, are overexpressed, or show allelic deletion. Although *neu* expression has been associated with comedocarcinomas, it is also found amplified in a number of other types of human breast cancers. The p21 protein of *ras* has also been associated with comedocarcinomas.[7] However, activated *myc* has not been associated with a specific tumor phenotype.

This systematic study verifies previous, more empirical, observations[4,9] and suggest that: 1) the histologic pattern of tumors is related to the particular oncogenes that are activated in tumors, 2) a genetic hierarchy controls the tumor phenotype, and 3) the oncogene or combination of oncogenes that are activated during tumorigenesis may effect the natural history of tumorigenesis. These observations have important implications in the interpretation of both animal and human tumors and begin to formulate the rules for understanding the relationship between tumor genotype and phenotype.

The mammary tumors of the transgenic mice showed both standard Dunn type and nonstandard histologic patterns. The greater percentage of tumors had histologically unusual patterns, and thus brought the whole concept of oncogene-specific tumor types to our attention. In the aforementioned study, we describe three types of tumors; eosinophilic small cell (SC), basophilic large cell (LC), and pale intermediate cell (IC), which were associated with the *ras, myc,* and *neu* oncogenes, respectively.

Each tumor phenotype was highly specific for the related oncogene and, in at least some cases, the presence of the particular oncogene or oncogene combination had a significant effect on the tumor incidence, kinetics and mean latency period. The most critical part of the

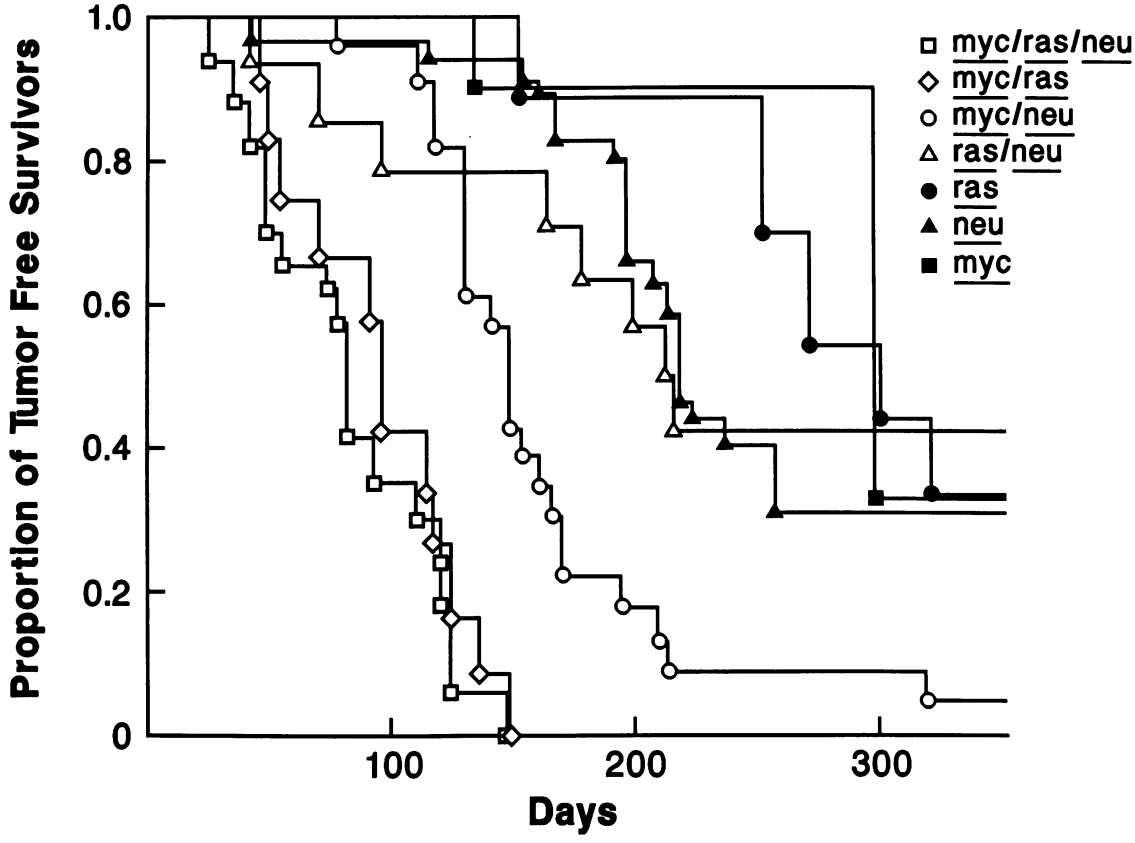

Figure 2. *The kinetics for mammary tumor occurrence in female mice from the TG.NK × TG.MSH cross. This graph is based on the total data file and not limited to tumors that are verified with slide examination. The individual populations are divided according to their actual genotype established by Southern blot.*

study was the analysis of the tumors in transgenic animals potentially carrying two or three of the oncogenes. In this analysis, the examiner had to classify the tumor without prior knowledge of the genotype. As in the over all analysis, the phenotype was highly specific for the genotype and had the same relative sensitivity (data not shown).

Of the three tumor types, the SC tumor had the highest association with its presumed oncogene, *ras*. However, this phenotype was also identified in animals with the *neu* oncogene. The histologic patterns of the IC and the SC tumors overlapped. The patterns found at each end of the continuum were clearly separable, so that the less frequent IC pattern was almost always correctly identified in the *neu*-bearing transgenics, and the SC tumor was highly specific for the *ras*-mice. On the other hand, the overlap was such that all of the *neu* animals in the trigenic crosses were judged to have tumors of the SC type. Apparently, the *neu*-related tumors do not develop as distinctive a phenotype as the other oncogenes and will have to be divided into subsets that are based on as yet undefined criteria.

The LC phenotype associated with the *myc* genotype proved to be the most dominant of the three phenotypes. When the *myc* allele was present in bigenic or trigenic animals, 75% of the tumors were of the basophilic large-cell type. Further, the SC type that was associated with the *ras* gene appeared to dominate the *neu* genotype. In a number of cases, an animal had multiple tumors. In some of these cases, the different tumors had different histologic patterns. These observations imply that when two or more oncogenes are activated in the same animal and same tissue, one will have more influence than others on the tumor phenotype. We have not found evidence that the shared genotype will cause the formation of a new tumor phenotype.

The oncogenes appear to produce characteristic patterns of tumor development. Although the patterns are compounded by the differences in the genetic backgrounds, it is clear that the combination of *myc* with either one or two of the other oncogenes increases the tumor incidence and reduces the mean tumor latent period. The fact that the combination of *ras* and *neu* actually reduced the tumor incidence indicates that tumorigenesis is dependent on the interactions of the oncogenes and not just the number of oncogenes that are activated

in the tumor. These observations suggest that the number and types of oncogenes in a tumor influence the biological behavior of the tumor.

Unfortunately, the data concerning oncogene expression in the tumors studied here is somewhat fragmentary and incomplete. However, re-examination of unpublished archival data suggests that *ras* expression is suppressed in many of the bigenic animals that also have the *neu* oncogene. Some data also imply that the temporal expression of one or more oncogenes is critical in determining the tumor type, which suggests a mechanism for the emergence of specific phenotypes from multigenic animals. These data, if verified, would effect our interpretation of the results, but more information is needed before a conclusion can be reached.

Finally, the morphologic evidence in this study suggests that the activation of a specific oncogene or combinations of oncogenes during tumorigenesis leads to specific molecular and biochemical events that dictate morphogenesis. Although most murine mammary tumors do not have morphologic counterparts amongst the common types of human breast cancer, the intermediate-cell tumor that was associated with the *neu* transgene in our mice has considerable resemblance to human comedocarcinomas. The association of the oncogenes with a specific histologic pattern in these animals has important implications to the study and classification of human tumors. Although some clear-out associations, such as comedocarcinoma of the breast and the presence of the HER-2 gene, have been described, a serious study of such associations needs to be undertaken.[4,6] It is intriguing to speculate that the surgical pathologist will someday be able to predict genotype and prognosis based on the tumor phenotype. Our studies begin to define the ground rules for recognizing critical relationships between tumor genotype and phenotype.

Acknowledgments

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