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SHORT COMMUNICATION

Pathogenetic characterization of 1-methyl-1-nitrosourea-induced mammary carcinomas in the rat

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The induction of mammary carcinogenesis in the rat by 1-methyl-1-nitrosourea (MNU) is widely used in experimental breast cancer research. In the experiments reported, the Ha-*ras* codon 12 (*ras*12) mutation (GGA→GAA) was used as a molecular marker to address issues of the clonality of carcinomas induced, pathogenetic independence among multiple carcinomas within the same animal and topographic distribution of mutant *ras*12 carcinomas in different mammary gland chains. In order to determine whether the frequently observed morphologically distinguishable lobules within carcinomas originate from the coalescence of independent lesions or whether cancerous cells within a carcinoma share a common origin, 44 randomly selected MNU-induced mammary carcinomas were genotyped for two to four lobules each for the *ras*12 mutation. A total of 43 carcinomas out of 44 (97.7%) had concordant *ras*12 genotypes among the multiple sites within each tumor, which is consistent with the latter possibility. Next, it was observed that as carcinoma multiplicity increased, the discordance rate of *ras*12 genotypes among multiple carcinomas within the same animal increased in a manner that was in excellent agreement with the expected discordance rate based on an assumption of no pathogenetic association among carcinomas. Furthermore, a significant difference was observed in the occurrence of mutant *ras*12 carcinomas between the cervical-thoracic and the abdominal-inguinal mammary glands in that three times as many carcinomas were mutant in the former as in the latter glands, whereas the occurrence of wild-type carcinomas was approximately the same in both regions. Taken together, the data are consistent with (i) carcinomas induced by MNU and detected by palpation are monoclonal in origin, (ii) independently-initiated cells emerge as distinct mammary carcinomas in the same animal, and (iii) the anatomical location of the gland may affect the prevalence of mammary carcinomas that harbor a mutant *ras*12.

The 1-methyl-1-nitrosourea (MNU*)-induced rat mammary carcinogenesis model (1) has contributed significantly to the current understanding of the biology of breast cancer and to potential approaches for its prevention. Major attributes of this model include that the proportion of mammary carcinomas that are ovarian-hormone dependent is similar to that observed in the human disease; that the carcinomas induced are aggressive and locally invasive; and that there is a clear operational distinction between the initiation and promotion stages of the disease process based on the action of MNU as a direct methylating agent (1–4). This latter feature of the model is often exploited to study effects of cancer preventive agents or risk factors on the promotion and progression stages of mammary carcinogenesis. Technical improvements since its original publication have made this model easier to implement and more reproducible (2,5,6). For example, Thompson and coworkers (5,6) have examined this model with respect to the route of carcinogen administration and have found that a single dose of MNU given intraperitoneally (i.p.) or subcutaneously (s.c.) was as effective as when it was given by intravenous (i.v.) injection, the method of administration originally reported (1). When MNU was administered by i.p. injection, smaller coefficients of variation in the number of carcinomas per rat were observed, an improvement the authors attributed to the consistent manner and the ease with which the MNU was delivered (6). The work reported here was based on the induction of mammary carcinogenesis by i.p. administration of MNU to female Sprague–Dawley rats at 50 days of age.

The pathogenetic characteristics of this experimental model of breast cancer are being defined with the use of molecular techniques. One of the identifiable somatic genetic changes is a GGA→GAA transition in Ha-*ras* proto-oncogene codon 12 (*ras*12) in a percentage of the carcinomas (7–9). Numerous studies have indicated that this mutation is an early initiating event (9–11) probably as a result of methylation of the guanine nucleosides (12,13), although some data have suggested that there might be a low frequency of spontaneous mutation of this codon in mammary epithelial cells (14). The early nature of the *ras*12 mutation in MNU-induced mammary carcinogenesis could therefore mark the initiated cells and their resultant carcinomas into two pathogenetic subpopulations, i.e. those with a mutant *ras*12 and those with a wild-type *ras*12 gene. Taking advantage of this mutation as a molecular marker, we addressed the following questions in order to gain further insights concerning the biology of the disease process in this model system:

1. Do the different morphologically discernible lobules that are frequently observed within mammary carcinomas (see examples in Figure 1A) indicate either that they arise from a coalescence of independent lesions (Figure 1B, Scheme 1) or that these lobules result from morphological diversification of clonally derived cells during tumor progression (Figure 1B, Scheme 2)?
2. Do multiple carcinomas within the same animal share the same pathogenetic characteristics such as *ras*12 mutation or do independently initiated foci of cells develop into distinct carcinomas?
3. Is the prevalence of mutant *ras*12 in carcinomas within an animal best modeled as a stochastic process or is there a bias based on the topographic location of the mammary gland from which a carcinoma arises?

Two animal experiments were conducted to provide the

*__Abbreviations:__ MNU, 1-methyl-1-nitrosourea; BW, body weight; H&E, hematoxylin and eosin; C-T, cervical-thoracic; A-I, abdominal-inguinal.

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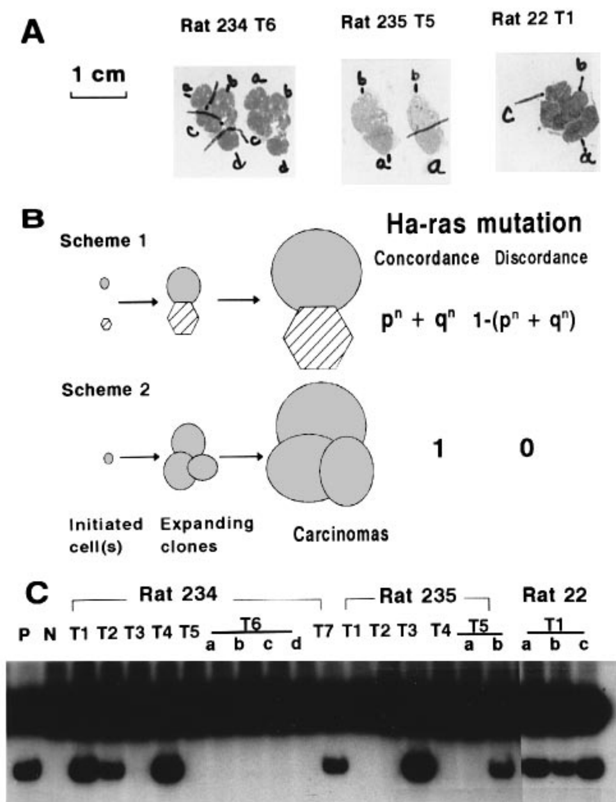

Fig. 1. (**A**) Examples of the gross appearance of tumor sections (H&E) on thin plastic slides. Tumors, especially large ones, were often observed to be made up of morphologically discernible lobules. The horizontal bar represents 1 cm in length. Sample code key: Rat234T6a, Rat #234, tumor #6, sampled site a. Lower case letters indicate sites from which tissue was sampled for *ras*12 genotyping. (**B**) Schematic illustration of multiple independent origins (polyclonality, Scheme 1) and a common origin of cancerous cells within a carcinoma (monoclonality, Scheme 2). (**C**) PCR-RFLP analysis of *ras*12 genotype in carcinomas. P, positive control for *ras*12 mutation. N, negative control for *ras*12 mutation, i.e. non-carcinogen treated rat mammary gland DNA. The presence of the shorter band is diagnostic of the *ras*12 mutation.

tissue samples for this study. Female Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY) at 21 days of age and fed a modified AIN76A diet. At 50 days they were given an i.p. injection of MNU (Ash Stevens Inc., Detroit, MI) by the method reported by Thompson and Adlakha (6). The dose level was 37.5 and 25 mg MNU per kg body weight (BW) for experiments 1 and 2 respectively. The rats were palpated for mammary tumors twice per week. When a tumor was first palpated, the date and the tumor location were recorded. The experiments were terminated at 22 and 25 weeks post-carcinogen for experiments 1 and 2 respectively. At necropsy, tumors and suspicious lesions were excised, fixed in 10% neutral buffered formalin (12 h) and later embedded in paraffin and sectioned for histological evaluation. The pathological criteria were as described by Young and Hallowes (15). Only tumors that were classified as carcinomas were used for genotyping the *ras*12 status.

The paraffin-embedded tumor blocks were serially cut into 5-μm sections and were mounted on thin transparent plastic slides coated with polylysine (Sigma Chemical Company, St Louis, MO) and stained with hematoxylin and eosin (H&E). Each section was viewed without a cover slip under light microscopy and marked into distinct lobules for tissue retrieval (see examples in Figure 1A). A small piece (~2×2 mm) was carefully cut with flame-sterilized scissors from each marked area. Each piece was incubated with 10 μg proteinase K in 50 μl of 100 mM Tris–HCl, 2 mM EDTA at 50°C for 3 h. After the proteinase K was inactivated by heating at 95°C for 8 min, 2–5 μl of the extract was used as the source of DNA for 40 cycles of PCR amplification.

The mutational status of *ras*12 was determined by a modified polymerase chain reaction-generated restriction fragment length polymorphism (PCR-RFLP) method (16,17). The upstream primer (5′AGTGTGATTCTCATTGGCAG-3′) was placed in intron-1 to avoid amplifying the Ha-*ras* pseudogene (17). The G→A mutation and two introduced mismatches in the downstream primer (5′-AGGGCACTCTTTCgaACGCC-3′, mismatches denoted by low case letters) generated an XmnI site in the PCR product (116 bp). Upon digestion of the product with XmnI (New England Biolabs, Beverly, MA), a fragment of 98 bp would be generated that was diagnostic for the mutation. A tracer amount of α-^{32}P-dCTP was used to label the PCR products. The digested products were separated by electrophoresis on a 6% polyacrylamide gel and detected by autoradiography as shown in Figure 1C.

Statistical methods used in the analyses of these experiments included descriptive statistics and χ^2-tests including Mantel–Haenszel tests for homogeneity of the association stratified by number of carcinomas per animal.

To address the first issue, 44 randomly selected carcinomas were analyzed. Of these carcinomas, 25 were sampled with two sites each, five with three sites each, and 14 with four sites each. Each site was genotyped for *ras*12 status (see examples in Figure 1C, rat234T6a-d, rat235T5a,b and rat22T1a-c). A total of 43 of 44 mammary carcinomas analyzed showed concordant *ras*12 (i.e. either all sites were mutant or all sites were wild type) among the multiple sites sampled (Table I). The exception was rat235 T5 in which the two sites were discordant for *ras*12. In fact, this observation initially prompted us to examine the issue of the origin of morphologically discernible lobules, which were often observed within carcinomas, especially in large ones.

It is of interest to note that the intensity of the diagnostic band varied considerably from carcinoma to carcinoma. Because the level of the mutant *ras*12 fraction in a sample can be influenced by the time frame of the occurrence of the mutation in relationship to carcinoma development, i.e. a mutation that occurred very late in the carcinogenesis process would be expected to result in a small mutant fraction in a tumor, the following factors were considered in the interpretation of these data. First, due to the stochastic nature of the carcinogenic initiation, the probability of mutating both *ras*12 alleles in the same initiated epithelial cell would be much lower than that of mutating only one allele. It was therefore expected that most of the mutant *ras*12 carcinomas would be heterozygous yielding at most a 50% mutant signal. In fact, out of >3000 MNU-induced mammary carcinomas analyzed so far in our laboratories, only two were observed to show a mutant *ras*12 signal that was >50% (unpublished data). Second, the percentage of non-epithelial cells in a tumor, which are less likely to harbor *ras*12 mutation, is quite variable among different carcinomas. Since the cancerous epithelial cells were not microdissected in this work, the inclusion of the non-epithelial cells would result in a varying degree of dilution of the mutant *ras*12 signal. Third, the carcinomas were fixed in formalin and DNA was extracted by proteinase K digestion and boiling. A varying degree of DNA damage could result from these

Table I. Ha-*ras* codon 12 genotyping of multiple lobules of randomly selected mammary carcinomas from experiment 1

Number of sites analyzed per carcinoma (n)	Predicted *ras* concordance rate among sampled sites		Observed number of carcinomas with		Observed *ras*12 concordance rate among sampled sites
	Assuming polyclonality[a]	Assuming monoclonality[b]	concordant *ras* among sampled sites	discordant *ras* among sampled sites	
2	0.505	1	24	1[c]	0.96
3	0.258	1	5	0	1
4	0.132	1	14	0	1
Total		1	43		0.977

[a]Predicted *ras*12 concordance rate based on multiple, independent origins for cells in different lobules within a carcinoma (see Figure 1B, Scheme 1). The probability by chance for say 3 sites to show the same mutant *ras*12 genotype is $p \times p \times p$ and to show wild-type *ras*12 genotype is $q \times q \times q$, where p = probability for mutant *ras*12 and $q = 1 - p$ = probability for wild-type *ras*12 at a given site. Thus the overall concordance probability = $p^3 + q^3$. For n sites sampled, the predicted concordance is calculated by formula $p^n + q^n$. p was estimated by the overall frequency of mutant *ras*12 carcinomas and in this experiment, $p = 0.45$.
[b]Predicted based on monoclonal origin. The discordant *ras*12 genotypes among different sites is 0 because all sites will be either wild type or mutant at codon 12. The concordant rate is independent of the number of sites (n) sampled.
[c]This section (Rat 235 Tumor 5) displayed two distinctly H&E-stained regions. The discordant *ras*12 genotypes of the two portions sampled indicated that this section represented two independently initiated carcinomas growing together side-by-side.

treatments and lead to less than perfect templates for PCR. Fourth, the Taq polymerase used for PCR has a low but detectable level of amplification error per base incorporated (~0.02% with 20 cycles), which involves predominantly A→G transitions (21). Because the detection of the diagnostic mutant signal relies on the XmnI enzyme to recognize a six-base restriction sequence (...GAA*nnnn*TTC...), any amplification error in that sequence as a result of these latter two factors would lead to resistance to enzyme digestion of the PCR products, further reducing the mutant signal intensity. It was therefore reasoned that mutant *ras*12 signal ranging from 5% to 50% would be consistent with this mutation being an early marker in MNU induced mammary carcinogenesis. The diagnostic band intensity observed in both experiments was within this range.

With these factors taken into consideration, the high degree of *ras*12 concordance among multiple sites within a carcinoma (97.7%) strongly support Scheme 2 (Figure 1B), i.e. morphological heterogeneity, often manifesting as distinct lobes within a carcinoma, is likely the result of diversification of progeny cells of the original initiated cell during clonal expansion and subsequent progression as a carcinoma develops. A practical implication of this information is that tissue sampling for genotyping purposes, at least as far as the *ras*12 mutation is concerned, can be achieved by a single sample per tumor with good accuracy.

Do multiple carcinomas within the same animal share the same pathogenetic characteristics? If the answer to this question is yes, it should follow that multiple carcinomas within an animal will display concordant *ras*12 genotype because all carcinomas are either all mutant or all wild type. As shown in Figure 1C (rat234, T1-T7 and rat235, T1-T5) this was not the case. Table II tabulates the observed *ras*12 discordance rate as a function of the number of carcinomas borne by a rat. The data are consistent with the probabilities predicted based on independent origins among multiple carcinomas within the same animal (as illustrated in Figure 1B, Scheme 1). The result was observed in two independent experiments in which different amounts of carcinogen were used to induce mammary carcinogenesis. The independent nature of individual carcinomas within an animal supports the use of carcinoma multiplicity as a parameter for assessing the effects of preventive agents as well as risk factors. It should be noted, however, that the independent nature of initiation inferred here is true only at the molecular marker level. Our data do not rule out physiological (i.e. epigenetic) interdependence among carcinomas within the same animal. Such an epigenetic interaction among carcinomas or initiated cells can potentially result from changes in the endocrine factors and metabolic milieu brought about by a preexisting carcinoma and could influence the emergence of additional carcinomas in the same animal and/or the latency of their appearance. In an early study with this model, the kinetics of appearance of additional carcinomas was observed to slow down significantly after the appearance of the first carcinoma (2). The implication of a secreted inhibitory factor from a primary tumor in suppressing the emergence of secondary tumors (18) might account for this observation.

To address the issue of topographic location of mutant *ras*12 carcinomas with respect to the mammary gland chains, Table III summarizes the prevalence of wild-type and mutant carcinomas arising in the cervical-thoracic (C-T) and the abdominal-inguinal (A-I) glands. A significant regional difference in total carcinoma occurrence was observed between the C-T and the A-I glands in that there were approximately twice as many carcinomas in the former as in the latter glands, which is consistent with previous reports (1,5,6,19). But surprisingly, more than three times as many mutant *ras*12 carcinomas were located in the C-T glands as in the A-I glands, whereas the wild-type *ras*12 carcinomas were almost equally distributed between the two regions (Table III). The disproportional distribution pattern held true upon secondary analyses stratifying by the number of carcinomas per animal and by experiment. In fact, the previously observed 2:1 C-T to A-I ratio of carcinoma occurrence (1,5,6,19) could be almost entirely attributed to this preferential localization of mutant *ras*12 carcinomas in the C-T mammary gland chains. Whether this difference is related to the asynchronous post-natal development of the C-T versus and A-I glands (19) remains to be determined. Nonetheless, the practical implication of the observed regional differences should not be overlooked. Until the cause and the biological significance of the regional differences observed in this study are clearly understood, it is advisable to follow a consistent tissue collection protocol with respect to carcinoma location in the mammary gland chains so that this source of bias is minimized when carcinoma tissues

Table II. Ha-*ras* genotype profile of multiple mammary carcinomas within the same animals

Number of carcinomas per rat (N)	Predicted *ras* discordance rate among multiple carcinomas assuming independent origin[a]	Number of rats with concordant *ras* genotypes among carcinomas	Number of rats with discordant *ras* genotypes among carcinomas	Total number of rats in category	Observed *ras* discordance rate among multiple carcinomas
Experiment 1 (37.5 mg MNU per kg)					
2	0.495	11	10	21	0.476
3	0.742	3	8	11	0.727
4	0.868	0	8	8	1
5	0.931	0	11	11	1
6	0.964	1	9	10	0.9
7 or greater	>0.981	0	17	17	1
Experiment 2 (25 mg MNU per kg)					
2	0.442	22	22	44	0.5
3	0.663	8	12	20	0.6
4	0.787	4	11	15	0.73
5	0.861	2	4	6	0.67
6 or greater	>0.908	0	3	3	1

[a]Predicted discordance rate among multiple carcinomas borne by the same animal assuming pathogenetic independence. Calculated by formula $1 - (p^N + q^N)$, where p was estimated by the overall *ras*12 mutation frequency in carcinomas. $p = 0.45$, $q = 1 - p = 0.55$ in experiment 1 and $p = 0.67$, $q = 0.33$ in experiment 2 respectively. N = number of carcinomas per rat.

Table III. Distribution of mutant and wild-type *ras*12 mammary carcinomas by anatomical regions

Location of glands	Number of carcinomas with		Total	% Ha-*ras* mutation	χ^2, P-value[a]
	mutant *ras*12	wild type *ras*12			
Experiment 1 (37.5 mg MNU per kg)					
Cervical-thoracic chains	132	115	247	53	
Abdominal-inguinal chains	41	91	132	31	
Total	173	206	379	45	17.4 ($P < 0.005$)
Experiment 2 (25 mg MNU per kg)					
Cervical-thoracic chains	171	61	232	74	
Abdominal-iguinal chains	54	51	105	51	
Total	225	112	337	67	16.1 ($P < 0.005$)

[a]2×2 contingency table analysis, degree of freedom = 1. The strong association between the anatomical region and occurrence of mutant *ras*12 carcinomas observed in both experiments were further examined by stratifying over the total number of carcinomas per animal and by experiment. The overall Cochran–Mantel–Haenszel $\chi^2 = 33$, $P < 0.001$. The disproportional pattern of mutant *ras*12 carcinoma occurrence was observed for each of the 10 strata in experiment 1, and 7 out of 8 strata in experiment 2. The probability for such observed disproportional distribution occurring by chance is $P < 0.01$. This secondary analyses did not support the existence of bias of the distribution pattern due to carcinoma multiplicity per animal.

are collected for biochemical and cytological assessment. The sampling issue is especially significant when 'gene-specific' prevention of subpopulations of pathogenetically identifiable neoplasia is concerned. For such applications of molecular markers, it is imperative that identifiable cancerous lesions from every gland be genotyped.

The overall *ras*12 mutation frequency in mammary carcinomas was 45% at a MNU dose of 37.5 mg/kg and 67% at 25 mg/kg (Table III). These results confirmed an earlier report that the percentage of mutant *ras*12 carcinomas was inversely related to the dose of MNU (10). That study also reported the disproportional increase in wild-type *ras*12 carcinomas in rats with experimental hyperprolactinemia (10). We have reported that the incidence of wild-type *ras*12 carcinomas could be increased preferentially by dietary risk factors over those with the mutation (17,20). These studies highlight the potential importance of risk assessment based on a knowledge of the pathogenetic characteristics of the disease.

In summary, experimental data presented in this study were consistent with the clonal evolution of multiple, independently-initiated cells giving rise to distinct mammary carcinomas in the same animal, and pointed to a significant topographic difference in the occurrence of mutant *ras*12 carcinomas between the C-T and the A-I mammary glands. These observations support the validity of statistical tests based on the assumption of independent emergence of lesions for the evaluation of the carcinogenesis data in this model and they further stress the need of representative sampling with gland location to be taken into consideration.

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