Genetic factors and suppression of metastatic ability of v-Ha-ras-transfected rat mammary cancer cells

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ABSTRACT Following v-Ha-ras transfection of nonmetastatic dimethylbenz[a]anthracene-induced rat mammary cancer (RMC1) cells, occasional transfectants were isolated that acquired high metastatic ability. High metastatic ability is not a simple process regulated by v-Ha-ras p21 levels alone in these v-Ha-ras transfecteds but involves the development of cytogenetic changes. If such cytogenetic changes involve only gain in gene expression, then all hybrids formed by fusing highly metastatic v-Ha-ras RMC1 transfectants with the parental nonmetastatic RMC1 should be highly metastatic. If loss of a metastatic suppressor gene(s) is also involved, then such hybrids should be nonmetastatic since chromosomes from the nonmetastatic parental cells should supply the suppressor function. To test this possibility, a highly metastatic cloned v-Ha-ras RMC1 transfectant was fused with the nonmetastatic parental RMC1 cells. Five hybrid clones were isolated that conserved the chromosomes from their parental cells. When these hybrid clones were injected into animals, primary tumors developed with the same tumor-doubling time as that of the highly metastatic parental v-Ha-ras transfected (i.e., ~2 days). High metastatic ability was, however, suppressed in these hybrid clones. All hybrid clones continued to express v-Ha-ras p21. Thus, suppression of metastatic ability in the hybrids can occur even in the presence of an elevated v-Ha-ras p21 level. This suggests that the acquisition of metastatic ability following v-Ha-ras transfection involves loss of metastasis suppressor gene function in rat mammary cancer cells.

Expression of the mutated Ha-ras oncogene or overexpression of the normal Ha-ras protooncogene stimulates proliferation and induces transformation in a number of cell lines (1). Previous studies have demonstrated that initially nontumorigenic NIH 3T3 cells can acquire both a tumorigenic phenotype and a high metastatic ability in nude mice following transfection with the mutated Ha-ras oncogene (2–7). The acquisition of metastatic ability following the Ha-ras oncogene transfection, however, is not universal and is dependent on type of the recipient cell (8).

It has been demonstrated that when nonmetastatic rat mammary cancer (RMC1) cells are transfected with the mutated Ha-ras oncogene, an occasional resultant transfectant develops high metastatic ability (9). There is, however, no simple dose-response relationship between the level of the mutated Ha-ras expression in these transfectants and the development of metastases. Cytogenetic analysis of the same RMC1 system has demonstrated that the frequency of chromosomal changes in the mutated Ha-ras transfecteds is significantly higher than that in control transfectants (10). These studies also suggest that if the appropriate chromosomal changes occur, these v-Ha-ras RMC1 transfectants acquire high metastatic ability. If such cytogenetic changes involve gain in gene expression only, then all hybrids formed by fusing highly metastatic v-Ha-ras RMC1 transfectants with the parental nonmetastatic RMC1 should be highly metastatic. If the loss of a metastatic suppressor gene(s) is also involved, then such hybrids should be nonmetastatic since chromosomes from the nonmetastatic parental cells should supply the suppressor function. To test this possibility, highly metastatic cloned v-Ha-ras RMC1 transfectant cells were fused with nonmetastatic parental RMC1 cells and the metastatic behavior of the hybrid clones was examined.

MATERIALS AND METHODS

Animals. All animals used in these studies were 6- to 8-wk-old female athymic nude mice obtained from Harlan-Sprague-Dawley. Tumor cells (5 x 10^6) were injected s.c. into nude mice and the tumor volume-doubling time was determined as described (11).

Cell Culture and Transfection of RMC1 Cells. A hormonally independent dimethylbenz[a]anthracene-induced rat mammary cancer (i.e., RMC1) was established in culture as described (9). This RMC1 cell line was maintained as a monolayer culture in RPMI 1640 medium/10% fetal bovine serum (HyClone), containing streptomycin (100 µg/ml), penicillin (100 units/ml), and dexamethasone (250 nM) (standard medium) at 37°C in 5% CO_2/95% air. RMC1 cells were transfected either with plasmid pY3, which encodes the hygromycin B-resistance gene (12), or with plasmid prasZip-6, which encodes the v-Ha-ras oncogene plus the neomycin-resistance gene (13) (i.e., coding for resistance to the G-418 neomycin analog) by the calcium phosphate precipitation procedure as described (14). Five independent clones were obtained from RMC1 cells transfected with plasmid pY3 and nine independent clones were obtained from RMC1 cells transfected with plasmid prasZip-6 using hygromycin B (500 µg/ml) selection and G-418 (500 µg/ml) selection, respectively, as described (14). One clone from RMC1 cells transfected with plasmid pY3 (RMC1-hygro) and one highly metastatic clone from RMC1 cells transfected with plasmid prasZip-6 (RMC1-Ras) were used to produce hybrid cells. These RMC1-hygro cells and RMC1-Ras cells were maintained in standard medium with hygromycin B (500 µg/ml; Gibco) and G-418 (500 µg/ml; Gibco), respectively.

Cell Hybridization. Nonmetastatic RMC1-hygro cells were fused with highly metastatic RMC1-Ras cells according to the method described by Davidson et al. (15). After 24 hr the standard medium was replaced with selective medium [i.e., standard medium containing hygromycin B at 500 µg/ml plus G-418 at 500 µg/ml (hygro + G-418 medium)] to select hybrid cells. Five hybrid clones (RMC1-hygro–RMC1-Ras hybrids 1–5) were randomly selected and were maintained in the hygro + G-418 medium.

Chromosomal Analysis. Chromosomal slides of the cultured cells were prepared as described (16). Chromosomes were banded using the trypsin/Giemsa technique (16). The number of chromosomes in a minimum of 50 G-banded (G-banded) cell spreads was counted for each cell clone. Karyotypes were constructed according to the scheme of Satoh et al. (17). At least 10 G-banded spreads were karyotyped for each cell line.
Table 1. Karyotypic analysis of RMC1, RMC1-hygro, RMC1-Ras, and RMC1-hygro-RMC1-Ras hybrid cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Modal chromosome no.</th>
<th>Normal chromosome</th>
<th>Structural change</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMC1 (parental)</td>
<td>44</td>
<td>2 2 2 2 2 2 3 2 2 2 3 2 2 2 2</td>
<td>1 1</td>
</tr>
<tr>
<td>RMC1-hygro</td>
<td>44</td>
<td>2 2 2 2 2 2 3 2 2 2 3 2 2 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 1 1 1 1</td>
</tr>
<tr>
<td>RMC1-Ras</td>
<td>42</td>
<td>1 2 2 1 2 2 3 2 2 2 2 2 2 2 2 2 2 2 2 2 2 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>RMC1-hygro-RMC1-Ras</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expected</td>
<td>86</td>
<td>3 4 4 3 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 2 2 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>Observed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrid 1</td>
<td>85</td>
<td>3 4 4 3 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 2 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>Hybrid 2</td>
<td>84</td>
<td>3 4 4 2 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 2 0 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>Hybrid 3</td>
<td>84</td>
<td>3 4 4 3 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 2 1 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>Hybrid 4</td>
<td>86</td>
<td>3 4 4 3 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 2 2 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>Hybrid 5</td>
<td>85</td>
<td>3 4 4 3 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 2 0 1 1 1 0</td>
<td></td>
</tr>
</tbody>
</table>

The highest number of each normal chromosome or structural change was determined. Structural changes (A–C) are described in Fig. 1. Values different from those expected for the RMC1-hygro–RMC1-Ras hybrid are underlined. Additional consistent structural changes in hybrid: 1, Yp+; 2, 9q+, Yp+; 3, 7q+; 5, 4p+, 9p+.

Spontaneous Metastasis Assay. The spontaneous metastatic ability was determined by inoculating $5 \times 10^5$ cells s.c. in one leg each of a series of nude mice. When the tumors became 1–2 g, the tumor-bearing legs were amputated while the animals were fully anesthetized with sodium pentobarbital (50 mg/kg). The mice were then allowed to go untreated until death or until 28 days after leg amputation. At autopsy, lung metastases were scored.

Western Blotting of p21 Ras. Western blot analysis to determine the level of mutated v-Ha-ras p21 expression for each cell clone was performed as described (9). Relative expression of p21 was normalized to the p21 level of the parental RMC1 cells.

Statistics. Metastatic ability of RMC1-hygro–RMC1-Ras hybrid clones was compared to that of the highly metastatic RMC1 Ras clone by using the Fisher exact test (18), which is sufficiently vigorous for use with small sample sizes. A difference was considered statistically significant at $P < 0.05$.

RESULTS

Cell Hybridization and Chromosomal Analysis. Parental RMC1 cells have a karyotype consisting of 44,XY,+7,+12 (10). RMC1-hygro cells had the same karyotype as parental RMC1 cells (Table 1 and Fig. 1A), whereas RMC1-Ras cells exhibited structural changes [del(1)(q41), 12q+, and der(15)(4;15)(q22;q24)] (Table 1 and Fig. 1B). RMC1-hygro cells were fused with RMC1-Ras cells, and five RMC1-hygro–RMC1-Ras hybrid clones (RMC1-hygro–RMC1-Ras hybrids 1–5) were randomly selected. Modal chromosomal numbers of these hybrid clones were 84–86 (Table 1). Karyotypic analysis showed that all five hybrid clones conserved most of chromosomes from their parental cells, demonstrating all of them were true hybrids (Table 1 and Fig. 2).

In Vivo Characteristics. When injected s.c. into nude mice, all the RMC1 clones and hybrids tested produced continuously growing tumors. The RMC1-Ras clone and the all five RMC1-hygro–RMC1-Ras hybrids produced tumors that grew at a significantly higher rate (approximately 4- to 5-fold) than those produced by the parental RMC1 clone or the RMC1-hygro clone (Table 2). When injected s.c. into the legs of nude mice, parental RMC1 cells and RMC1-hygro cells produced no lung metastases, whereas RMC1-Ras cells produced lung metastases in all animals injected (Table 2). All five RMC1-hygro–RMC1-Ras hybrids had significantly less metastatic

![Fig. 1](https://example.com/fig1.png) Representative karyotypes of RMC1-hygro (A) and RMC1-Ras (B) cells. Large arrowheads indicate numerical abnormalities and small arrowheads (β) indicate structurally rearranged chromosomes: A, del(1)(q41); B, 12q+; C, der(15)(4;15)(q22;q24).
ability than the RMC1-Ras clone, although these hybrids and the RMC1-Ras clone had the same in vivo growth rate, which was 4- to 5-fold higher than that of the nonmetastatic parental RMC1 clone or the RMC1-hygro clone (Table 2).

**Ha-ras p21 Expression.** The p21 encoded by the Ha-ras oncogene was identified in all the RMC1 clones and hybrids by Western blot analysis using a monoclonal anti-ras p21 antibody that detects both normal and mutated forms of p21 Ras protein. As shown in Fig. 3, parental RMC1 cells and RMC1-hygro cells express only the normal form of p21 while the RMC1-Ras cell clone and all five hybrids express a doublet in the p21 region. The viral Ha-ras oncogene encodes both phosphorylated (upper band) and unphosphorylated (lower band) forms of p21, but the cellular ras gene encodes only the unphosphorylated form of p21 (19). Quantitation of the Ha-ras p21 revealed that RMC1-hygro cells and RMC1-Ras cells expressed 0.2- and 10-fold the p21 level of the parental RMC1 cells. All of the hybrids continued to express high p21 levels (3- to 5-fold) compared to parental RMC1 cells. These levels in the hybrids were approximately one-third to one-half those of the RMC1-Ras cells.

**DISCUSSION**

The present studies demonstrate that hybrid cells produced by fusing slow-growing nonmetastatic rat mammary cancer cells and fast-growing highly metastatic v-Ha-ras-transfected rat mammary cancer cells have the same in vivo tumor growth rate as the fast-growing highly metastatic v-Ha-ras-transfected cells. In contrast, high metastatic ability, however, is suppressed in these hybrid clones. Cytogenetic analysis demonstrates that all hybrid clones retain most of the chromosomes from their parental cells. All hybrid clones continue to express v-Ha-ras p21, although the expression is at a slightly lower level from that of the parental v-Ha-ras-transfected clone. Previous studies have demonstrated that although v-Ha-ras transfection can result in the development of metastatic ability in the rat mammary cancer cells, there is no simple dose-response relationship between the level of v-Ha-ras expression and the acquisition of high metastatic ability among cloned rat mammary cancer cell transfecants (9). For example, v-Ha-ras transfectants that express a p21 level 1- to 2-fold higher than that of the parental non-v-Ha-ras-transfected rat mammary cancer cells have a high metastatic ability, while other v-Ha-ras transfectants that express a 6- to 10-fold higher p21 level have a low metastatic ability (9). Thus the slight decrease in p21 level in the hybrids as compared to v-Ha-ras parental cells does not appear to be sufficient to explain the suppression of metastatic ability.

Further studies on the same system have demonstrated that the acquisition of high metastatic ability involves chromosomal changes resulting from genetic instability following v-Ha-ras transfection (10). Such chromosomal changes involve both gains and losses of a variety of chromosomal material (10). If the acquisition of high metastatic ability due to the genetic instability induced by v-Ha-ras expression was simply the result of gains in expression of various oncogenes, then the hybrids should retain these positive changes and be

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**Table 2. Characteristics of RMC1, RMC1-hygro, RMC1-Ras, and RMC1-hygro-RMC1-Ras hybrid cells**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Animals, no.</th>
<th>Tumor doubling time, days</th>
<th>% of group with lung metastases</th>
<th>Lung metastases per animal, no.</th>
<th>Relative expression of Ha-ras p21</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMC1 (parental)</td>
<td>8</td>
<td>11.6 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>RMC1-hygro</td>
<td>8</td>
<td>9.9 ± 1.2</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>RMC1-Ras</td>
<td>8</td>
<td>2.3 ± 0.1</td>
<td>100</td>
<td>13 ± 7</td>
<td>10</td>
</tr>
<tr>
<td>RMC1-hygro-RMC1-Ras</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrid 1</td>
<td>5</td>
<td>2.2 ± 0.1</td>
<td>20*</td>
<td>0.4 ± 0.4</td>
<td>5</td>
</tr>
<tr>
<td>Hybrid 2</td>
<td>5</td>
<td>2.0 ± 0.2</td>
<td>0*</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Hybrid 3</td>
<td>5</td>
<td>2.4 ± 0.2</td>
<td>20*</td>
<td>0.4 ± 0.4</td>
<td>5</td>
</tr>
<tr>
<td>Hybrid 4</td>
<td>5</td>
<td>2.2 ± 0.2</td>
<td>40*</td>
<td>0.8 ± 0.5</td>
<td>5</td>
</tr>
<tr>
<td>Hybrid 5</td>
<td>5</td>
<td>2.6 ± 0.1</td>
<td>0*</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

*Ha-ras p21 expression is relative to that of the parental RMC1 cells. Tumor doubling times are expressed as mean ± SEM.*

*P < 0.05 vs. RMC1-Ras.
fusion studies with cells. The monoclonal lesion has a metastatic recombinant Ras protein. The mutated v-Ha-ras proteins migrated with lower mobilities than the normal protein, indicating the mutated lesion at position 12 of the native p21 molecule. Each lane contained $\approx 100 \mu g$ of protein.

highly metastatic. The fact that these hybrids are not metastatic is thus inconsistent with positive changes alone being responsible for the acquisition of high metastatic ability in this system. Alternatively, these results suggest that the suppression of high metastatic ability in hybrid clones is due to the replacement of the expression of a metastasis suppressor gene(s) provided by the nonmetastatic parental clone.

Indirect evidence for the existence of a metastasis suppressor gene(s) has also been demonstrated by several cell fusion studies (20–24). In these studies, metastatic potential was suppressed when rat metastatic mammary carcinoma cells were fused with various nonmetastatic cells (20), when mouse metastatic melanoma cells were fused with normal cells (21, 22), when mouse metastatic lung carcinoma cells were fused with tumorigenic but nonmetastatic mouse L cells (23), and when highly metastatic rat prostate cancer cells were fused with nonmetastatic rat prostate cancer cells (24). Metastatic phenotypes of the parental cells in these studies, however, were spontaneously developed. The present studies demonstrate that suppression of metastatic ability in the hybrid cells can occur even in the presence of an increased v-Ha-ras p21 level in rat mammary cancer cells. This suggests that the acquisition of metastatic ability following v-Ha-ras transfection involves loss of metastasis suppressor gene function in this system.

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