HMGIC gene expression is not required for in vivo thyroid cell transformation

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Introduction

The HMGIC protein family comprises three members: HMGIC, HMGII and HMGY. The last two proteins are products of alternative splicing of the same gene, named HMGIC(Y) (1), the HMGIC protein is coded for by a related gene (2). The HMGIC proteins bind DNA and participate in the assembly of protein complexes on the promoters of several inducible genes, thus they are defined as architectural transcription factors (3–5).

The HMGIC genes are abundantly and, almost ubiquitously, expressed during embryogenesis (6,7), and are absent or expressed at low levels in adult mouse and human tissues (2,7). HMGIC gene overexpression was first described in rat thyroid transformed cells and in experimental thyroid tumors (8–10). Further studies assessed HMGIC overexpression as a common feature of experimental and human malignant neoplasias (11–17).

To determine whether overexpression of HMGIC was important for tumorigenesis or a result of cell transformation, HMGIC expression was blocked by introducing an antisense HMGIC cDNA expression construct into rat thyroid cells. When these cells were subsequently infected by Myeloproliferative sarcoma virus (MPSV) and the Kirsten murine sarcoma virus (KiMSV) carrying the v-mos and v-ras-Ki oncogenes, respectively, they were unable to grow in soft agar or to form tumors when injected into athymic mice. Conversely, untransfected rat thyroid cells after retroviral infection formed colonies in soft agar and were highly tumorigenic after injection into athymic mice. Interestingly, in these experiments expression of HMGIC(Y) was totally abolished at the RNA level, raising the possibility that blocking transformation also secondarily inhibited expression of these related family members (18). These results suggested that the induction of HMGIC-C, and/or HMGIC(Y), expression is required in the process of thyroid cell transformation.

Therefore, we aimed to verify whether the HMGIC-C gene has a pivotal role in in vivo thyroid neoplastic transformation, evaluating the susceptibility of HMGIC-C null mice for developing thyroid malignancies. In fact, transgenic insertional mice with the pygmy phenotype were generated (19). Analysis of the integration locus revealed that it occurred in the HMGIC-C locus. Then, mice carrying a disrupted HMGIC-C gene were produced (6). These mice also showed a pygmy phenotype with craniofacial defects and decrease in fat tissue with no alteration in the growth hormone–insulin-like growth factor endocrine pathway. The phenotype shown by the HMGIC-C–/– and mutant insertional mice was very similar to spontaneous dwarf mice (pg), described previously (20), in which both the HMGIC-C alleles are deleted (6).

Therefore, spontaneous HMGIC-C mouse pygmy mutants (pg) were either treated with radioactive iodine (131I) or crossed with the TgE7 mice, which carried the E7 gene of the papilloma virus, under the transcriptional control of bovine thyroglobulin gene promoter. The TgE7 mice developed a well-differentiated thyroid carcinoma at 9–11 months of age (21).

Here, we show that the frequency of radiation- and E7-induced thyroid carcinomas was the same in pg and wild-type mice, indicating that HMGIC-C is not required for in vivo thyroid malignant transformation. On the basis of the previously published data (18) and the HMGIC(Y) gene overexpression in all of these carcinomas, we suggest that the expression of HMGIC(Y) is sufficient for in vivo thyroid carcinogenesis.

Materials and methods

Animals

Transgenic mice carrying the papilloma type 16 E7 transgene under the transcriptional control of bovine thyroglobulin gene promoter (TgE7) have been described previously (21). Transgenic mouse TgE7 has been described previously (21). Spontaneous mutant pg mice have also been described previously (20) and were obtained from The Jackson Laboratory (Bar Harbor, ME).

Experimental thyroid carcinogenesis

For the induction of radiation-induced thyroid neoplasias, 5–8-week-old C3H, homozygous (pg–/–) and heterozygous (pg+/–) spontaneous pygmy mutant mice were treated with 131I at the dose of 10 µCi by intraperitoneal injection. Then 0.5% propyl-thiouracil was added to the drinking water until animals were killed after 50–61 weeks.

Generation of TgE7/pg+/- and TgE7/pg–/– mice

TgE7 mice in a C57 genetic background, were bred for five generations in a C3H genetic background to reduce the strain-related genetic effect on tumor formation.
development. They were then crossed with C3H pygmy +/− mice to generate the TgE7/pg−/− mice. Since homozygous pg−/− mice were unfruitful, the TgE7/pg−/− were crossed with each other to generate the homozygous TgE7/pg−/− mice.

Southern blot analysis

Southern blotting was performed according to a standard procedure (22). DNA was extracted from tail biopsies and the genomic DNA was digested. For the screening of TgE7 mice, DNA was digested with BamHI. The blots were hybridized with a labeled probe corresponding to the 2.0 kb fragment of the bovine thyroglobulin promoter (21). The screening for the pygmy genotype was performed by digestion of genomic DNA with EcoRI. The blots were hybridized with a labeled probe corresponding to the 1.7 kb CDNA of mouse HMGI-C (2).

RT−PCR analysis of the expression of HMGI(Y) and HMGI-C

Total RNA was extracted by RNeasy (Tel-Test, Inc., Friendswood, TX). Total RNA, digested with DNase, was reverse transcribed using random exonucleotides as primers (100 mM) and 12 U AMV reverse transcriptase ( Gibco) and subsequent PCR amplification was performed as reported previously (23). cDNA (200 ng) was amplified in a 25 µl reaction mixture containing Taq DNA polymerase buffer, 0.2 mM dNTPs, 1.5 mM MgCl2, 0.4 mM of each primer, 1 U Taq DNA polymerase (Perkin Elmer). The PCR amplification was performed for 30 cycles (94°C for 30 s, 55°C for 2 min and 72°C for 2 min). For the detection of the HMGI(Y) and HMGI-C specific sequences, the primers used were: 5’−AGGAGGAATGCGAGGTG−3’ and 5’−CGGGTTCTGGGTTGCCTGG−3’ (corresponding to nucleotides 197−214 and 421−438, respectively, of the HMGI(Y) cDNA); and 5’−GGTACCGGTTAAGCAGTAG−3’ and 5’−ACATGTTTCAATGATTC−3’ (corresponding to nucleotides 47−66 and 507−526, respectively, of the HMGI-C cDNA). Amplification of the constitutively expressed enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) was performed as an internal control for the amount of CDNA tested. The GAPDH specific primers were forward: 5’−GACATGGTCAATGATTC−3’ and reverse: 5’−TGGACTCCACAGTCTCAG−3’ (corresponding to nucleotides 336−356). The products of the reactions were analyzed on a 2% agarose gel, and then transferred by electroblotting to GeneScreen plus nylon membrane (Du Pont, Boston, MA). DNA was fixed to the membranes by air-drying and UV crosslinking, then membranes were hybridized with the specific probes.

Histological and immunohistochemical procedures

For light microscopy, tissues were fixed by immersion for 24 h in Bouin’s solution and embedded in paraffin using standard procedures. Sections (5 µm) were stained with hematoxylin and eosin or hematoxylin and periodic acid–Schiff (PAS) reagent. Frozen sections (4 µm) of normal and pathological tissues were cut in a frozen microtome and allowed to dry for 1 h at room temperature, before fixing in acetone for 10 min. The slides were air dried for 2 h at room temperature and then placed in a buffer bath (phosphate buffered saline, PBS) for 5 min before the immunoperoxidase staining procedure.

For the immunohistochemical studies of paraffin-embedded samples, 3−4 µm paraffin sections were deparaffinized and then placed in a solution of absolute methanol and 0.5% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining.

The slides were then incubated overnight at 4°C in a humidified chamber with the antibodies diluted 1:100 in PBS. The slides were subsequently incubated with biotinylated goat anti-rabbit IgG for 20 min (Vectastain ABC kits, Vector Laboratories) and then with preimmixed reagent ABC (Vector) for 20 min. The immunostaining was performed by incubating the slides in diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 min. After chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent-mounting medium (Permount). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system.

The antibodies used in this study were raised against the synthetic peptide SSSKQQLASKQ specific for the HMGI(Y) proteins (14). For the HMGI-C immunoreactivity, the antibodies used in this study were raised against the recombinant HMGI-C protein (18).

The specificity of the reaction was validated by the absence of staining when carcinoma samples were stained with antibodies pre-incubated with the peptide against which the antibodies were raised (data not shown). Similarly, no positivity was observed when tumor samples were stained with a pre-immune serum (see Figure 3B).

Results

Pygmy (pg−/−) and wild-type (pg+/+) mice showed the same susceptibility to radiation-induced thyroid carcinomas

Eleven pg−/−, 20 pg+/− and 18 C3H mice were treated with radioactive iodine and then a goitrogenic agent as described in Materials and methods. The animals were killed within 40−60 weeks of treatment and their thyroids were examined. The results of the histological examination of the thyroid nodules that developed in these animals indicate that no differences, in terms of carcinoma frequency and histological features, were observed among the different groups (Table I and Figure 1A and B). These results suggest that the HMGI-C gene is not required for in vivo radioiodine-induced thyroid malignant cell transformation.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Hyperplasia</th>
<th>Adenomas</th>
<th>Papillary carcinomas</th>
</tr>
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<tbody>
<tr>
<td>C3H</td>
<td>8</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>pg−/+</td>
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Thyroid targeted expression of HPV E7 oncogene induces thyroid carcinomas in pygmy mice (pg−/−) as well as in wild-type (pg+/+) mice

It has been reported previously that transgenic mice expressing the E7 papilloma virus oncogene under the transcriptional control of thyroglobulin develop differentiated and functionally regulated thyroid goiters. Old mice display secondary tumor nodules that mimic the various histological aspects of the human differentiated thyroid cancer (21). pg−/− mice expressing the E7 transgene under the transcriptional control of the thyroglobulin bovine promoter (TgE7−pg−/−) were generated. Sixteen pg−/−/TgE7, 16 pg−/+/ TgE7 and 15 TgE7 mice were bred. Two mice from each group were killed at 6 and 9 months, and the thyroids examined. At 12 months, all of the remaining mice were killed. The results of the histological diagnoses of the removed thyroids are summarized in Table II.

At 6 months, nodules characterized by high mitotic rates were observed. Within 9 months we observed the appearance of nodules characterized by cytological and architectural disorders such as micropapillary architecture developed within a macrofollicular area. At 12 months, a large majority of the animals carrying the E7 gene developed a generalized thyroid hyperplasia and hypertrophy with the presence of focal papillary lesions, characterized by cytologic aspects found in the papillary subtype of human thyroid carcinomas, i.e. nuclear polymorphism with nuclear grooves and ground glass cells. However, no differences were observed in the three different groups in tumor development (Table I) or histological aspects (Figure 1C−F).

Expression of HMGI(Y) and HMGI-C genes in the thyroid carcinomas induced in the pygmy mice

HMGI genes are induced in thyroid cell transformation in vitro and in vivo (8−10,14), and the expression of the HMGI-C gene is required for in vitro thyroid cell malignant transformation (18). As such, we analyzed the expression of the HMGI genes in the neoplastic thyroid samples originating from wild-type and pygmy mice that had either been treated with radioactive iodine or crossed with the TgE7 mice.

Immunohistochemical analysis of the thyroid carcinoma samples did not show expression of HMGI-C in thyroid carcinomas of pygmy mice (Figure 2B and D), while HMGI-C induction was observed in all of the carcinomas from the...
Thyroid carcinomas in pygmy mice

Fig. 1. Thyroid carcinoma in pigmy (pg−/−) and wild-type (pg+/+) mice. (A and B) Radiation-induced thyroid carcinomas in pigmy (pg−/−) and wild-type (pg+/+) mice. (C and D) 20× Thyroid carcinomas in mice expressing the HPV E7 oncogene in pigmy (pg−/−) and wild-type (pg+/+) mice, respectively. (E and F) As above, at a higher magnification.

<table>
<thead>
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<th>Hyperplasia</th>
<th>Adenomas</th>
<th>Papillary carcinomas</th>
</tr>
</thead>
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wild-type C3H (Figure 2C and E) and the heterozygous mice (data not shown). As far as the HMGI(Y) gene expression was concerned, the induction was observed in all of the carcinoma samples derived from C3H (Figure 3C), homozygous (pg−/−) (Figure 3D), heterozygous (pg+/−) (data not shown), TgE7/pg−/− (Figure 3E) and TgE7 mice (Figure 3F). No staining for HMGI-C and HMGI(Y) was detected in normal thyroid of C3H mice (Figure 2A and 3A, respectively). The specificity of the reaction was validated by the absence of staining when a neoplastic section was incubated with a pre-immune serum (Figure 3B) or with antibodies pre-incubated with the peptide against which antibodies were raised (data not shown).

The same carcinoma samples were analyzed by RT–PCR. The results are shown in Figure 4. They parallel those obtained by immunohistochemistry. HMGI-C and HMGI(Y) overexpression was observed in all the carcinoma samples, apart from the absence of the HMGI-C expression in the mice carrying a disrupted HMGI-C genes in both the alleles.

Table II. Thyroid lesions detected in C3H and pigmy mice carrying the TgE7 transgene
Fig. 2. Immunohistochemical detection of HMGI-C in thyroid carcinomas. Paraffin sections from normal thyroid and carcinomas were analyzed by immunohistochemistry using antibodies raised against the recombinant HMGI-C protein. (A) Immunostaining of a normal thyroid (20×) using antibodies raised versus HMGI-C. No immunoreactivity was observed. (B and D) Immunostaining of a thyroid papillary carcinoma in pg−/− treated with 131I (B) or in pg−/−TgE7 (D). No nuclear staining was observed. (C and E) Immunostaining of a thyroid papillary carcinoma in pg+/+ treated with 131I (C) or in pg+/+TgE7 (E). Nuclear staining was observed.

**Discussion**

HMGI proteins are overexpressed in all of the malignant neoplasias so far analyzed (11–17). We previously demonstrated that HMGI protein overexpression has a causal role in thyroid cell transformation, since blocking the HMGI-C synthesis by an antisense methodology prevents the acquisition of the malignant phenotype induced in thyroid cells by the MPSV and KiMSV virus. The lack of induction of AP-1 transcripational activity by suppression of HMGI synthesis may account for inhibition of the neoplastic cell transformation in absence of HMGI proteins. In fact, thyroid neoplastic transformation was associated with a drastic increase in AP-1 activity, which was blocked by the suppression of the HMGI protein synthesis. The absence of AP-1 transcripational activity induction, directly or indirectly regulated by the HMGI proteins, would inhibit the expression of AP-1-dependent genes, such as VEGF, collagenase I and stromelisin, which are required to achieve cell neoplastic transformation (24).

To assess the role of the HMGI-C gene in *in vivo* thyroid cell transformation, spontaneous pygmy mice, in which both the HMGI-C alleles have been deleted, were treated with radioactive iodine. It is worth noting that previous experiments have shown a high frequency of *Ki-ras* activation in radiation-induced thyroid tumors (25). The animals treated developed thyroid papillary carcinomas with the same efficiency and without any significant morphological differences either in the presence or absence of the HMGI-C protein. The same results were obtained when the pygmy mice were crossed with the TgE7 mice: the hybrid mice continued to develop papillary carcinomas.

The tumors induced in pygmy and wild-type mice were analyzed for the expression of the HMGI genes. Papillary carcinomas from the pg−/− mice were obviously negative for the expression of HMGI-C showing the induction of the HMGI(Y) gene. Conversely, in the pg+/+ mice both the HMGI genes were expressed at high levels.

In conclusion, the results presented here indicate that the HMGI-C is not necessary for thyroid carcinogenesis *in vivo*. Several hypotheses can be envisaged to explain this discrepancy with our published data showing that HMGI-C is required for *v-mos-* and *v-ras-Ki*-induced cell transformation *in vitro* (18). The models used do not correspond perfectly, and we could hypothesize that radiation-induced transformation might follow a different pathway, which is HMGI-C-independent, and that E7, a nuclear oncogene, may work downstream of the HMGI proteins, even though induction of the HMGI(Y) proteins has been found in all the carcinomas induced by iodine or by the
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