Heterogeneous Nuclear Ribonucleoprotein G Shows Tumor Suppressive Effect against Oral Squamous Cell Carcinoma Cells

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Abstract

Purpose: Heterogeneous nuclear ribonucleoproteins (hnRNP) are nucleic acid binding proteins involved in RNA processing. We found that hnRNP G is expressed in normal human oral epithelial cells while frequently not found in the cells derived from human oral squamous cell carcinomas (HOSCC). The current study was designed to test the hypothesis that hnRNP G is a tumor suppressor.

Experimental Design: We investigated the expression levels of hnRNP G protein in normal, precancerous, and malignant oral tissues by in situ immunohistochemistry. In addition, wild-type or mutant hnRNP G was ectopically overexpressed in HOSCC cells and their effects on cellular replication kinetics, colonogenic efficiency, anchorage-independent growth, and in vivo tumorigenicity were determined.

Results: In situ immunohistochemical staining showed robust presence of hnRNP G in the basal cell layers of normal oral epithelium but the level of its staining was markedly reduced in dysplastic or cancerous tissues. Ectopic expression of wild-type hnRNP G in cancer cells lacking hnRNP G expression or containing mutant hnRNP G resulted in severe retardation of proliferation, reduction of colonogenic efficiency, loss of anchorage-independent growth, and reduction of in vivo tumorigenicity in immunocompromised mice. In addition, hnRNP G overexpression led to up-regulation of the expression of TXNIP, a cell cycle inhibitory gene, and significantly reduced the expression of the genes that promote cellular proliferation, such as EGR1, JUND, JUNB, FOS, FOSL1, ROS, and KIT.

Conclusions: These results indicate that hnRNP G is a tumor suppressor against HOSCC but its mechanisms of action remain to be further investigated.

Heterogeneous nuclear ribonucleoproteins (hnRNP) constitute a large family of nucleic acid-binding proteins with more than 30 different members. hnRNPs were first described as chromatin-associated RNA binding proteins with the major role in RNA processing (1). However, recent studies showed that the biological functions of hnRNPs are extremely diverse and include RNA turnover, telomere biogenesis, oncogenesis, and spermatogenesis (2). For example, hnRNP D (AUF1) regulates the turnover rate of α-globin mRNA by associating with the mRNA stability complex (3). hnRNP types D, A1, and C1/C2 interact with human telomerase holoenzyme (4–6) and are involved in telomere elongation (7). Furthermore, hnRNP B1 is recognized as a biomarker for early detection of lung cancer (8). The hnRNP types A1 and E2 are overexpressed during leukemogenesis and affect the proliferation, survival, and differentiation of normal and leukemic cells (9). hnRNP G, encoded by the RBMX locus found in chromosome X, plays an important role in spermatogenesis along with the RBMY gene product and hnRNP G-T, which interact with Tra2β, an activator of pre-mRNA splicing in spermatocytes (10). Besides its prescribed function in RNA splicing and spermatogenesis, hnRNP G is one of the least characterized protein among the members of hnRNPs for its biological functions.

hnRNP G was first identified as a nuclear protein with apparent molecular weight of 43 kDa (11, 12). It is composed of 391 amino acid residues encompassing the RNA binding domain (amino acids 10-88) at the NH2 terminus of the protein (11). This structural feature suggests the physical association of hnRNP G with RNA, which may be required for its role in RNA processing and metabolism (1, 11, 13). hnRNP G is detected primarily in the nuclei of mammalian cells and localized on lambrush chromosomes of amphibian oocytes, supporting its association with nascent RNA transcripts as part of the transcriptional complexes (11). A recent study showed that hnRNP G alters pre-mRNA splicing pattern by antagonizing the
effects of Tra2β, a splicing activator (14). Therefore, the level of hnRNP G expression and its biochemical activities may have profound and multifaceted effects on the global gene expression profile in cells and maintenance of normal cellular homeostasis.

The current study was undertaken to investigate the possible involvement of hnRNP G deregulation in human oral carcinogenesis. We initially observed that the overexpression of hnRNP G in human oral squamous cell carcinoma (HOSCC) cells led to reduction in cell proliferation capacity and viability. Furthermore, hnRNP G expression was diminished in the cultured HOSCC cells whereas it was readily detectable in replicating normal human oral keratinocytes. hnRNP G expression in situ was also not detected in the oral epithelial tissues showing dysplastic or cancerous histopathology whereas its expression was found in the basal cell layers of normal oral epithelium. Ectopic expression of hnRNP G in HOSCC cells, such as HEp-2 and SCC4, resulted in notable inhibition of anchorage-independent growth capacities and tumorigenicity in vivo. These findings indicate the tumor-suppressive effects of hnRNP G against HOSCC cells.

Materials and Methods

Cells and culture conditions. Primary normal human oral keratinocytes were prepared from separated epithelial tissue and serially subcultured in Keratinocyte Growth Medium (Cambrex, East Rutherford, NJ) containing 0.15 mmol/L Caclus as previously described (15). The SCC4, SCC9, SCC15, and HEp-2 cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD) and the Tu-139 cancer cell line was kindly provided by Drs. G. Clayman (University of Texas Medical Center, Houston, TX), SCC4, SCC9, and SCC15 were cultured in DMEM/Ham’s F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Woodland, CA) and 0.4 μg/mL hydrocortisone (Sigma, St. Louis, MO) whereas Tu-139 was cultured in DMEM/Ham’s F12 supplemented with 10% fetal bovine serum. HEp-2 was grown in MEM (Invitrogen) supplemented with 10% fetal bovine serum.

Western blot analysis. Whole-cell extracts were isolated from semi-confluent cultures of normal human oral keratinocytes and the HOSCC cell lines as previously described (15). Protein concentration was determined using Protein Assay Reagent (Bio-Rad, Hercules, CA). One confluent culture of normal human oral keratinocytes and the HOSCC cell lines were selected with 100 μg/mL G418 using Qiagen RNeasy total RNA isolation kit (Qiagen, Chatsworth, CA). cDNA was synthesized from 10 μg total RNA and the biotin-labeled antisense cRNA was produced by in vitro transcription using the ENZO BioArray HighYield kit. cRNA (15 μg) was fragmented and hybridized to Affymetrix HG-U133 Plus 2.0. The chip was stained with streptavidin-phycocerythrin and analyzed using GCOS 1.2 software for pairwise comparison of gene expression. The statistical significance for each gene was evaluated by ANOVA single-factor analysis using Microsoft Excel 2000 and the fold difference >3.0, as well as P < 0.05, was considered significant.

Reverse transcription-PCR. Total RNA isolated from the HEP-2 cells infected with LXSX-hnRNP G or LXSX was reverse transcribed using SuperScript First-Strand Synthesis System (Invitrogen). The following genes were analyzed by reverse transcription-PCR: TXNIP, IL-8, ATF3, EGR1, JUND, JUNB, KIT, FOS, and FOSS1. Glyceraldehyde-3-phosphate dehydrogenase cDNA was also amplified to control for the starting cDNA amount. The primers for each gene were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers were designed to specifically amplify the interexonic sequences to rule out the possibility of amplifying the contaminating genomic DNA, although such a possibility is highly unlikely. The primer sequences will be available on request. First-strand cDNA (2 μL) was amplified in a 50-μL PCR reaction volume containing 1× PCR buffer [20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl], 2 mmol/L MgCl2, 0.2 mmol/L each deoxynucleotide triphosphate, 0.2 μmol/L each primer, and 2 units of Platinum Taq Polymerase (Invitrogen) under the following conditions: initial denaturation at 94°C for 2 minutes, followed by 20, 25, or 30 amplification cycles with
denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. A 5-μL volume of each PCR reaction was then loaded onto 1% agarose gels stained with ethidium bromide.

Results

hnRNP G expression is down-regulated in cells and tissues of HOSCC. To determine the association between the expression levels of hnRNP G with HOSCC, we first investigated the expression levels of this protein in normal human oral keratinocytes and five different cancer cell lines (i.e., HEp-2, SCC4, SCC9, SCC15, and Tu-139) derived from HOSCC. hnRNP G expression was readily detectable in normal human oral keratinocytes by Western blotting but was significantly diminished in all tested HOSCC cells, some of which completely lacked the expression (Fig. 1A). The protein expression was also assessed in formalin-fixed, paraffin-embedded normal oral mucosa (n = 7), precancerous dysplastic oral lesions (n = 9), and invasive cancer (n = 11) by in situ immunohistochemistry (Table 1). A representative staining pattern is shown in Fig. 1B, which illustrates hnRNP G expression in normal, dysplastic, and cancerous epithelium obtained from a single biopsy specimen. The hnRNP G expression was confined primarily to the basal cell layer (stratum basale) of normal oral epithelium with intense intranuclear and weak cytoplasmic staining. However, the dysplastic and cancerous epithelium showed weak or undetectable hnRNP G staining. Two HOSCC specimens (specimen nos. 4 and 6 of the HOSCC category, Table 1) exhibited moderate hnRNP G expression but the staining was localized in the cell membrane without any intranuclear hnRNP G staining (see Fig. 1B).

Table 1. hnRNP G immunoreactivity in situ is diminished in oral epithelium with dysplastic and cancerous histopathology

<table>
<thead>
<tr>
<th>Pathologic category</th>
<th>Specimen no.</th>
<th>Histopathologic finding</th>
<th>hnRNP G*</th>
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<tbody>
<tr>
<td>Normal (n = 7)</td>
<td>1</td>
<td>Normal oral epithelium</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Normal oral epithelium</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<td>+++</td>
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<tr>
<td></td>
<td>4</td>
<td>Normal oral epithelium</td>
<td>+++</td>
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<tr>
<td></td>
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<td>Normal oral epithelium</td>
<td>+++</td>
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<tr>
<td></td>
<td>6</td>
<td>Normal oral epithelium</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Normal oral epithelium</td>
<td>+++</td>
</tr>
<tr>
<td>Dysplasia (n = 9)</td>
<td>1</td>
<td>Moderate-severe epithelial dysplasia</td>
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<tr>
<td></td>
<td>2</td>
<td>Focal keratosis and mild epithelial dysplasia</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Mild epithelial dysplasia</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
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<td>-</td>
</tr>
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<td>Moderate-severe epithelial dysplasia</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Mild epithelial dysplasia</td>
<td>+</td>
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<tr>
<td></td>
<td>7</td>
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<td>+</td>
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<tr>
<td></td>
<td>8</td>
<td>Severe epithelial dysplasia</td>
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<tr>
<td></td>
<td>9</td>
<td>Severe epithelial dysplasia</td>
<td>+</td>
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<td>HOSCC (n = 11)</td>
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<td>-</td>
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<td>2</td>
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<td></td>
<td>4</td>
<td>Superficial moderately differentiated squamous cell carcinoma</td>
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<td></td>
<td>5</td>
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<tr>
<td></td>
<td>11</td>
<td>Moderately differentiated squamous cell carcinoma</td>
<td>-</td>
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</table>

*The level of hnRNP G immunostaining per each specimen was scored as negative (−), weak (+), moderate (++), or strong (+++) by two independent observers, noting the level of chromogenic development after addition of the 3,3′-diaminobenzidine substrate.

† These HOSCC specimens exhibited moderate (++) hnRNP G staining in the membrane but lacked intranuclear staining (see Fig. 1B).

Unpublished observation.
LXSN-hnRNP-K22R, or the insertless vector (LXSN), and selected with 100 μg/mL G418. Colonogenic efficiency of the infected cells was determined after 9 days postinfection by Giemsa staining of the culture flasks. The culture infected with LXSN or LXSN-hnRNP-K22R showed replicating colonies of varying sizes whereas the culture infected with LXSN-hnRNP G did not contain any visible colonies (Fig. 2A). In addition, the replication kinetics of the cells expressing hnRNP G was notably repressed compared with the cells infected with LXSN or LXSN-hnRNP-K22R (Fig. 2B).

The capacity of mammalian cells to form colonies in soft agar is closely correlated with their tumorigenic potential (20–22). Therefore, to assess the effect of hnRNP G on the malignant properties of HOSCC cells, we compared the ability of the parental HEp-2 and SCC4 cells and of those infected with LXSN, LXSN-hnRNP G, or LXSN-hnRNP-K22R to form colonies in soft agar. After 3 to 4 weeks, the colony-forming efficiencies of the tested cell types were examined by counting the colonies containing >50 cells. Both HEp-2 and SCC4 cells infected with LXSN-hnRNP G showed a significant reduction in the colony-forming efficiency in soft agar compared with the controls (i.e., parental cells and those infected with LXSN or LXSN-hnRNP-K22R; Table 2; Supplementary Fig. S1). These results indicate the inhibitory effects of hnRNP G on replication of the HOSCC cells in semisolid medium as well as in monolayer.

**Exogenous hnRNP G expression reduces tumorigenicity of HEp-2 cells in vivo.** The above results raised the possibility that hnRNP G possesses tumor-suppressive effects against HOSCC. This possibility was tested in our next experiment in which we determined the effects of hnRNP G on the tumorigenic potential of HEp-2 cells in vivo. HEp-2 cells infected with LXSN, LXSN-hnRNP G, or LXSN-hnRNP-K22R were selected with 100 μg/mL G418. The drug-resistant cells were injected s.c. into nude mice and the volumes of the resulting nodules were measured to determine the kinetics of tumor growth in vivo. The parental HEp-2 cells and those infected with LXSN or LXSN-hnRNP-K22R showed 71% (5 of 7), 57% (4 of 7), and 91% (10 of 11) efficiency for the same period of observation. The cells infected with LXSN-hnRNP G showed significantly reduced (30%; 3 of 10) efficiency for the same period of observation. The palpable nodules that developed from each cell group were harvested and examined histologically by H&E staining (Supplementary Fig. S2). The nodules that formed by the control HEp-2 cells and those expressing mutant hnRNP G were composed mainly of the malignant cells showing increased nuclear to cytoplasmic ratio, nuclear pleomorphism, and hyperchromatic nuclei. However, abundance of nonviable cells was noted in the nodule formed by the HEp-2 cells expressing exogenous wild-type hnRNP G. Taken together, the above results indicate the growth inhibitory effects of hnRNP G on the HOSCC cells and tumors in vitro and in vivo.
Identification of the genes differentially expressed by hnRNP G. It was possible that hnRNP G overexpression would alter the expression of the genes that triggered the observed growth inhibitory effects against the HOSCC cells. This possibility was investigated in our experiments in which we compared the global gene expression profiles between the HEp-2 cells infected with either LXSN or LXSN-hnRNP G using microarray-based gene expression analysis. The result confirmed that hnRNP G was greatly up-regulated (6.5-fold) in the HEp-2 cells expressing exogenous hnRNP G compared with the cells infected with LXSN. We identified 21 genes that were differentially expressed in cells overexpressing hnRNP G (Table 3). Among them, we have selected nine genes to confirm their differential regulation by exogenous hnRNP G expression.
Tumor-Suppressive Effect of hnRNP G

Fig. 3. Differential expression of genes by overexpression of hnRNP G in HEp-2 cells. Microarray analysis of the genes differentially expressed by ectopic expression of hnRNP G in HEp-2 cells was confirmed by semiquantitative reverse transcription-PCR. Total RNA was isolated from the parental HEp-2 cells and the cells infected with LXSN or LXSN-hnRNP G. Total cellular cDNA was synthesized by reverse transcription and the cDNAs of TXNIP, IL-8, EGR1, ATF3, JUND, JUNB, KIT, FOS, and FOSL1 were amplified by PCR using unique primer sets. PCR products were electrophoresed in 1% agarose gel and visualized by ethidium bromide staining.

(Fig. 3). Semiquantitative reverse transcription-PCR was done with total RNA isolated from the parental HEp-2 cells and from those infected with LXSN-hnRNP G or LXSN. Infection with LXSN alone did not alter their expression level when compared with those of the parental HEp-2 cells but the expression of exogenous hnRNP G led to differential expression of the tested genes. Consistent with the microarray data, the HEp-2 cells expressing exogenous hnRNP G showed marked induction of TXNIP and down-regulation of IL-8, ATR3, EGR1, JUND, JUNB, KIT, FOS, and FOSL1 expression when compared with the control cells. These results also support the growth inhibitory effects of hnRNP G on the cells and tumors of HOSCCs.

Discussion

We report for the first time the growth inhibitory effects of hnRNP G against HOSCC cells in vitro and in vivo. The cells and tissues of HOSCC exhibited aberrant expression of hnRNP G. This was first evinced by Western blotting, which revealed diminished hnRNP G protein level in the cell lines derived from HOSCCs compared with that of normal human oral keratinocytes. hnRNP G expression in situ was readily detectable in the replicating cells of normal basal epithelium but was absent in most of the tissue specimens showing aberrant histopathology. Absence or negligible amount of hnRNP G in all tested precancerous dysplastic tissues is very interesting because it indicates that the loss of hnRNP G expression may be involved in the early process of carcinogenesis. The determination of hnRNP G expression status in tissue could be useful for the early diagnosis of human oral carcinogenesis. Ectopic expression of hnRNP G in the HOSCC cells led to the loss of colonogenic efficiency, diminished replication kinetics, and inability to form replicating colonies in soft agar. Furthermore, exogenous hnRNP G expression significantly reduced the tumorigenic potential of the HOSCC cells in nude mice. Therefore, based on these results, we propose that hnRNP G is a tumor suppressor protein against HOSCC.

There were two HOSCC specimens with moderate level of hnRNP G expression detectable by immunohistochemistry, but the protein staining was primarily membranous, completely lacking intranuclear hnRNP G signal in these specimens. Thus, subcellular localization of hnRNP G as well as the intracellular expression level is altered in HOSCC cells. Recent studies showed the significance of nuclear localization signal of hnRNPs in compartmentalization of the protein in cells and apparently in their physiologic functions (23, 24). It is possible that the two HOSCC specimens with extranuclear hnRNP G expression pattern harbor mutations within the nuclear localization signal of the protein, leading to the loss of its tumor-suppressive function. The reason for the membranous localization of hnRNP G in the tested cancer tissue remains to be investigated.

Alkan et al. (25) recently showed that the mutations within the RNA substrates that could alter the binding affinity of hnRNPs led to the changes in gene expression. This finding indicates that the RNA binding capability of hnRNPs is critical for its effects on gene regulation. We found a naturally occurring intragenic missense mutation within the coding region of RBMX at codon 22 to yield a Lys-to-Arg transition (K22R). The K22R mutation of hnRNP G failed to inhibit cellular replication of the HOSCC cells in monolayer or in soft agar, and the HEp-2 cells harboring the mutant hnRNP G developed rapidly growing tumors in nude mice. Thus, this is the first report of a loss-of-function mutant within the RBMX coding region present in the HOSCC cells. Interestingly, the HEp-2 cells expressing the mutant hnRNP G showed a higher frequency of tumor development in vivo compared with the parental HEp-2 cells and those infected with LXSN (Table 2). It is possible that the mutant hnRNP G has growth-promoting activity through yet unknown mechanism. Protein domain mapping study showed the presence of ribonucleoprotein consensus RNA binding domain at the NH2 terminus of hnRNP G, including codon 22 (11). Thus, the mutant hnRNP G, which has lost its RNA binding capacity and tumor inhibitory effects, may fail to modulate the expression of its target genes. Conversely, it is possible that the wild-type hnRNP G elicits the tumor-suppressive effects by altering the expression of its downstream genes. This possibility was investigated by comparing the genome-wide gene expression profiles of the HEp-2 cells with or without the overexpression of exogenous hnRNP G.

The microarray data allowed us to identify the discrete gene groups that were differentially regulated by hnRNP G. First, TXNIP was greatly induced in the HEp-2 cells expressing hnRNP G. The expression of TXNIP, also known as VDUP1, correlates with the replication state of cells and is found to alter the cellular redox state (26). In addition, overexpression of exogenous TXNIP inhibits tumor cell growth and cell cycle progression via modulation of p21^{kip1} stability (27, 28). Second, the expression of several proto-oncogenes and those
which promote cellular proliferation [e.g., JUND (29), JUN (30, 31), FOS (31), JUNB (32), FOSL1 (33), KIT (34), and ROS (35)] were inhibited in the HEp-2 cells expressing hnRNP G. Third, the expression of IL-8, which was found to be a specific biomarker of HOSCC found in human saliva (36, 37), was down-regulated by hnRNP G overexpression in HEp-2 cells. Thus, the identity of the genes differentially regulated by hnRNP G is consistent with the notion supporting the tumor-suppressive effects of this protein. However, the microarray data also suggest the complexity of the molecular events occurring during the onset of cell growth arrest mediated by hnRNP G overexpression. Tumor suppression by hnRNP G is therefore likely to involve the multitude of molecular alterations that require further investigation.

References

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