

Growth Hormone-Releasing Hormone Receptor Splice Variant 1 is Frequently Expressed in Oral Squamous Cell Carcinomas

Nikolina Dioufa · Elena Farmaki · Andrew V. Schally · Hippokratris Kiaris · Dimitris Vlahodimitropoulos · Athanasios G. Papavassiliou · Christos Kittas · Norman L. Block · Ioulia Chatzistamou

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Abstract The expression of growth hormone-releasing hormone (GHRH) splice variant 1 (SV1) receptor in neoplastic lesions of the oral cavity was assessed. The sensitivity of HaCaT keratinocytes to GHRH analogs was also evaluated. Thirty-three benign precancerous oral lesions and 27 squamous cell carcinomas of the oral cavity were evaluated by immunohistochemistry for SV1 expression. SV1 expression in HaCaT keratinocytes was assessed by western blot. HaCaT proliferation was evaluated by cell counting. Anti-

SV1 immunoreactivity was detected in only 9 % (three of 33) precancerous lesions (one hyperplasia and two dysplasias), while 44 % (12 of 27) carcinomas were positive for SV1 ($p < 0.002$). GHRH(1–29)NH₂ and GHRH agonist JI-38 stimulated HaCaT proliferation in vitro, and this effect was blocked by GHRH antagonists. These results indicate that SV1 expression may be associated with the transition of precancerous lesions to carcinomas of the oral epithelium. GHRH antagonists may be useful for the management of the disease.

N. Dioufa · E. Farmaki · H. Kiaris · A. G. Papavassiliou
Department of Biological Chemistry,
University of Athens Medical School,
M. Asias 75,
115 27 Athens, Greece

A. V. Schally · N. L. Block
Endocrine, Polypeptide and Cancer Institute, Veterans Affairs
Medical Center and South Florida Veterans Affairs Foundation
for Research and Education,
1201 N.W. 16th Street,
Miami, FL 33125, USA

A. V. Schally · N. L. Block
Department of Pathology, University of Miami,
Miller School of Medicine,
1800 N.W. 10th Ave,
Miami, FL 33136, USA

A. V. Schally
Divisions of Hematology/Oncology and Endocrinology,
Department of Medicine, University of Miami,
Miller School of Medicine,
1800 N.W. 10th Ave,
Miami, FL 33136, USA

D. Vlahodimitropoulos
Department of Forensic Medicine and Toxicology,
University of Athens Medical School,
M. Asias 75,
115 27 Athens, Greece

C. Kittas
Laboratory of Histology and Embryology,
University of Athens Medical School,
M. Asias 75,
115 27 Athens, Greece

I. Chatzistamou (✉)
Department of Basic Sciences,
Dental School, University of Athens,
Thibon 2,
11527 Athens, Greece
e-mail: ihatzist@med.uoa.gr

Introduction

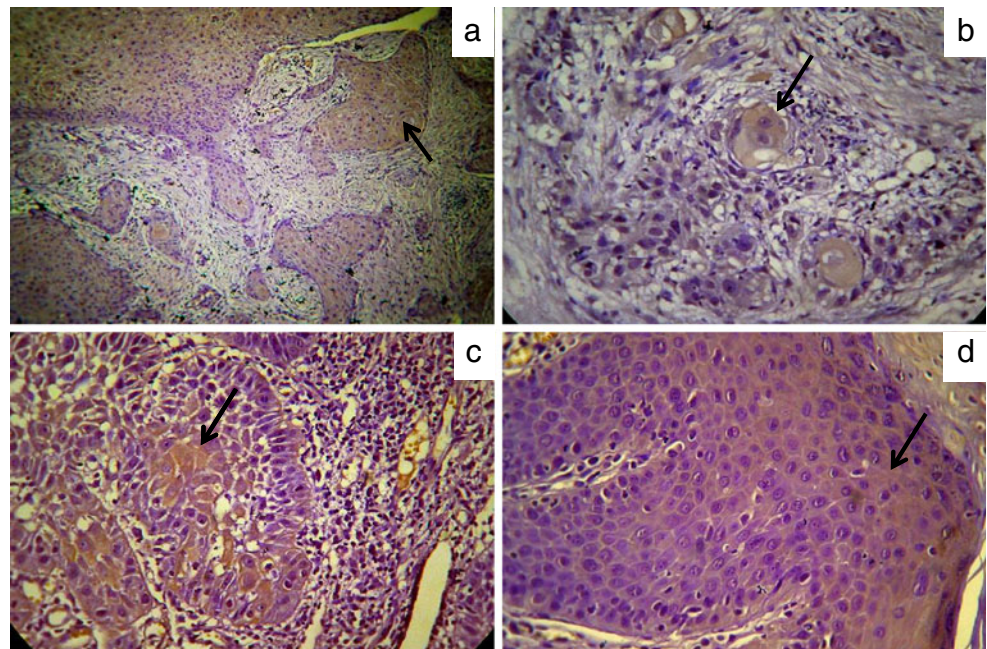
Oral cancer is among the ten most common cancers in the world, with broad differences in geographic distribution [1, 2]. The major risk factors for oral neoplasms are chronic exposure of oral mucosa to consumption of tobacco and alcohol [3, 4]. The most common type of oral cancer is squamous cell carcinoma (SCC) with dysplasias being considered as the histological precursor lesions for this malignancy [5]. Several molecular alterations have been associated with development of oral cancer including aberrant expression of p53, epidermal growth factor receptor, matrix metalloproteinases, *Ras* oncoproteins, and others [6–15]. In certain cases, these molecular changes may reflect etiology and ethnic origin [16]. A link between the development of oral cancer and human papilloma virus infection has also been proposed [17, 18]. Besides these autonomous cellular lesions, recent evidence has also revealed an important role for stroma activation in development of oral cancer [19–21]. Despite the progress made in the identification of the molecular alterations that are linked to oral SCC (OSCC) and their potential susceptibility to the development of targeted therapies [22, 23], the identification of additional alterations that are causally linked to the disease is imperative.

Growth hormone-releasing hormone (GHRH) is a neuroendocrine peptide hormone which regulates the production and release of GH in the pituitary and in peripheral tissues plays various roles that range from stimulation of cardiac and pancreatic cell to wound

healing and tissue repair [24–27]. In addition, overexpression of GHRH by various neoplastic tissues has also been reported. Importantly, the inhibition of autocrine GHRH activity by specific GHRH antagonists which has been found to inhibit cancer cell growth in vitro and in vivo underlines the potential use of these peptide analogs as a novel class of anticancer drugs [28–30]. The pituitary type of GHRH receptors and its splice variants (SVs) in the peripheral tissues and in the malignant tumors are likely to mediate the effects of GHRH. Among them, SV1 possesses the greatest similarity to pituitary GHRH receptor and has been shown to be expressed in several primary human tumors and many human cancer cell lines [31–36]. SV1 corresponds to a truncated form of GHRH receptor lacking a segment of the extracellular portion of the receptor [30]. However, it remains functional as it binds GHRH analogs and elicits mitogenic signals upon GHRH stimulation [37]. In addition, SV1 also possesses activity that is independent of GHRH ligand stimulation as its expression is sufficient to stimulate cell proliferation in the absence of GHRH [38, 39].

In the present study, we evaluated the expression of anti-SV1 immunoreactivity in a bank of primary oral lesions including squamous cell carcinomas and dysplasias. We also evaluated the effects of GHRH agonists and antagonists in vitro in HaCaT keratinocytes. Our results suggest that oral cancers express receptors for GHRH more commonly than do dysplasias and suggest that therapy with GHRH antagonists should be considered for the therapy of oral SCCs.

Fig. 1 Representative microphotographs of SV1 immunopositivity (brown staining indicated by *arrow*) in primary oral neoplastic lesions. **a** Well-differentiated SCC that is characterized as +++ for SV1 expression ($\times 20$ magnification). **b** Well-differentiated SCC that is characterized as + for SV1 expression ($\times 40$ magnification). **c** Well-differentiated SCC that is characterized as ++ for SV1 expression ($\times 40$ magnification). **d** Moderate epithelial dysplasia that is characterized as ++ for SV1 expression ($\times 40$ magnification)



Materials and Methods

Specimens and Immunohistochemistry

A bank of 60 oral tissue specimens consisting of 27 squamous cell carcinomas and 33 precancerous lesions (five hyperplasias and 28 dysplasias) was collected from the archives of the Laboratory of Histology and Embryology, University of Athens Medical School and was analyzed by immunohistochemistry for SV1 expression by using the antibody JH2317/5 [32]. The local Institutional Review Board for use of human subjects at Athens University approved the protocol for collection and use of these specimens and authorized the study. Human pituitary tissue of normal adult individuals was obtained from the tissue archives of the Department of

Forensic Medicine and Toxicology. Immunostaining was performed by using the Superpicture Polymer (Dab) Kit (Novocastra), following the manufacturer's instructions. Before evaluations, a weak counterstaining with hematoxylin was performed. Specimens were evaluated for positive immunohistochemical staining and classified according to the percentage of positive cells, into the following categories: 1–10 %, ±; 11–30 %, +; 31–70 %, ++; and 71–100 %, +++.

Peptides and Cell Culture

hGHRH(1–29) NH₂ was obtained from Sigma. Agonist JI-38 [40] and GHRH antagonists JMR-132 and MIA-602 were synthesized as reported [28, 41, 42]. The peptides were dissolved in phosphate buffered saline at the indicated

Table 1 Clinicopathological data and anti-SV1 immunoreactivity in pre-neoplastic oral lesions

Specimen no.	Gender	Age	Anatomical location	Histopathology	SV1
1	M	68	Floor of the mouth	Mild epithelial dysplasia	+
2	M	71	Gingiva	Mild epithelial dysplasia	–
3	F	68	Buccal mucosa	Mild epithelial dysplasia	±
4	M	72	Gingiva	Mild epithelial dysplasia	±
5	F	53	Lower lip	Epithelial hyperplasia	±
6	F	57	Buccal mucosa	Mild epithelial dysplasia	–
7	F	78	Tongue	Epithelial hyperplasia	++
8	F	76	Alveolar ridge	Mild epithelial dysplasia	++
9	M	53	Lower lip	Severe epithelial dysplasia	++
10	F	52	Gingiva	Moderate epithelial dysplasia	±
11	F	67	Tongue, gingiva	Moderate epithelial dysplasia	–
12	M	35	Tongue	Mild epithelial dysplasia	–
13	F	42	Palate	Epithelial hyperplasia	+
14	M	58	Tongue	Mild epithelial dysplasia	–
15	F	70	Tongue	Mild epithelial dysplasia	–
16	M	50	Floor of the mouth	Mild epithelial dysplasia	–
17	F	53	Gingiva	Mild epithelial dysplasia	–
18	M	62	Tongue	Mild epithelial dysplasia	–
19	M	38	Tongue	Mild epithelial dysplasia	+
20	F	42	Tongue	Mild epithelial dysplasia	–
21	F	49	Tongue	Mild epithelial dysplasia	–
22	F	53	Gingiva	Mild epithelial dysplasia	–
23	F	63	Buccal mucosa	Mild epithelial dysplasia	±
24	F	69	tongue	Mild epithelial dysplasia	–
25	M	58	Buccal mucosa	Mild epithelial dysplasia	±
26	F	38	Palate	Mild epithelial dysplasia	±
27	F	53	Tongue	Mild epithelial dysplasia	–
28	F	52	Buccal mucosa	Moderate epithelial dysplasia	+
29	F	60	Tongue	Mild epithelial dysplasia	–
30	F	58	Buccal mucosa	Mild epithelial dysplasia	–
31	M	34	Tongue	Epithelial hyperplasia	–
32	M	58	Tongue	Moderate epithelial dysplasia	
33	M	29	Gingiva	Epithelial hyperplasia	+

concentrations. Controls contained solvent alone. HaCaT cells were maintained in Dulbecco's modified Eagle medium containing 10 % fetal bovine serum (FBS) and antibiotics/antimycotics. Tissue culture reagents were obtained from Invitrogen.

Cell Proliferation and Migration Assays

The rate of cell proliferation was evaluated by cell counting under inverted microscope. Dead cells were identified by the Trypan blue exclusion staining and were not included in the analysis; 10^4 cells were seeded per well and were treated for 5 days with the peptides at the indicated concentrations. Cell migration was evaluated by the "scratch" assay as described, exposing cells to the peptide analogs for 24 h [43]. All in vitro experiments were performed in triplicates and yielded similar results.

Western Blot Analysis for SV1

HaCaT cells were seeded at a level of 30–40 % of confluence and cultured for 3 days in order to achieve a low-

density cell culture. For the high-density cell culture, cells were seeded at about 90 % confluence and cultured subsequently for 3 days prior to protein isolation. Total proteins were extracted from cultured HaCaT cells as previously described [43]. Briefly, the cells were lysed at 4°C for 20 min using a lysis buffer (radioimmunoprecipitation assay, Invitrogen, and proteinase inhibitor). Whole cell lysates were subsequently centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatants were collected. Protein content in the supernatants was determined by the Bradford assay. One hundred-microgram aliquots of the proteins extracted from cells were electrophoresed through a 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. The proteins were electrophoretically transferred from gels onto nitrocellulose membranes. The blots were exposed overnight to primary antibodies, followed by 1 h of incubation with secondary antibodies. The antigen–antibody complexes were detected with the enhanced chemiluminescence detection system (Thermo Scientific). The experiments were repeated with at least three different cultured specimens

Table 2 Clinicopathological data and anti-SV1 immunoreactivity in OSCC

Specimen no.	Gender	Age	Anatomical location	Histopathology	SV1
1	M	73	Buccal mucosa	Superficially invasive	++
2	M	55	Tongue	Moderate differentiated	++
3	M	96	Alveolar ridge	Well differentiated	++
4	M	63	Tongue	Well differentiated	++
5	M	82	Tongue	Well differentiated	–
6	F	35	Tongue	Well differentiated	–
7	M	48	Soft palate	Moderately differentiated	++
8	M	77	Tongue	Well differentiated	–
9	M	60	Tongue	Superficially invasive	–
10	F	NA	Tongue	Well differentiated	++
11	F	40	Lower lip	Well differentiated	+++
12	M	32	Tongue	Moderately differentiated	–
13	F	70	Tongue	Well differentiated	++
14	F	59	Tongue	Well differentiated	–
15	M	73	Soft palate	Well differentiated	++
16	F	54	Tongue	Well differentiated	–
17	M	78	Tongue	Well differentiated	++
18	F	NA	Alveolar ridge	Well differentiated	–
19	M	NA	Palate	Well differentiated	–
20	F	68	Palate	Moderately differentiated	–
21	M	69	Tongue	Well differentiated	++
22	F	53	Tongue	Well differentiated	–
23	M	72	Tongue	Well differentiated	–
24	M	48	Lip	Moderately differentiated	–
25	F	55	Lip	Moderately differentiated	–
26	M	49	Lip	Moderately differentiated	–
27	F	52	Alveolar ridge	Well differentiated	+++

with similar results, and the reported results are representative. Antibody for actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and SV1

antibody is the same as was used in immunohistochemistry [32].

Statistical Analysis

Immunohistochemical data were analyzed by the chi-squared test while the statistical analysis of tissue culture experiments was by the two-tailed Student's *t* test.

Results

SV1 Expression in Oral Cancers and Dysplasias

We have evaluated by immunohistochemistry the expression of the GHRH receptor SV1 in a bank of 60 neoplastic lesions of the oral cavity that included 33 precancerous lesions (five hyperplasias and 28 dysplasias) and 27 OSCCs. SV1 immunopositivity was detected in 15 specimens and was cytoplasmic in all cases (Fig. 1). In all cases, immunopositivity was localized in the neoplastic epithelial cells and was undetectable in the stromal cells of the tumors. Those positive (positivity $\geq ++$) for SV1 comprised 12 of 27 (44 %) OSCCs as compared to only three of 33 (9 %) precancerous lesions ($p < 0.002$) (Tables 1 and 2). Thus, expression of SV1 is significantly increased in oral neoplastic lesions as they progress into malignancy. Among the three precancerous lesions in which SV1 immunoreactivity was detected, two were dysplasias and one was hyperplasia. This implies that prior to malignant degeneration, expression of SV1 is not increased in dysplasias as compared to the hyperplasias despite that the former histopathological entity is considered to be the precursor of OSCC. No correlation between SV1 expression and the differentiation level or anatomic site of OSCCs was observed. As a control for

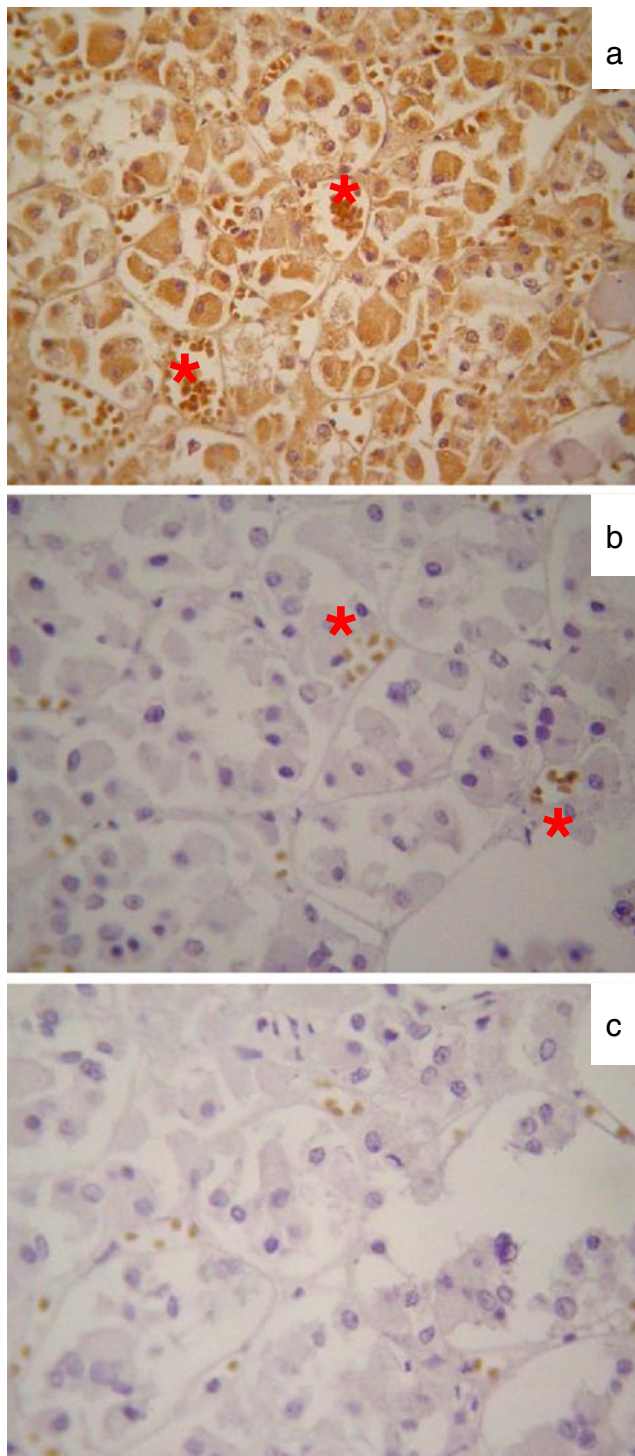


Fig. 2 Representative microphotographs of SV1 immunopositivity (brown staining) in normal human pituitary in the presence (a) or absence of the primary anti-SV1 (b) or secondary (c) antibody. Red asterisks in both panels indicate non-specific immunopositivity that developed against red blood cells

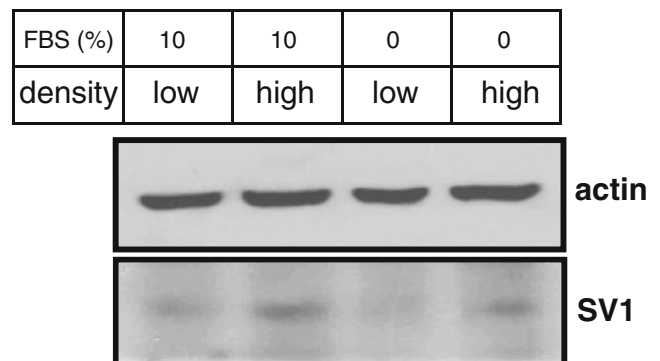
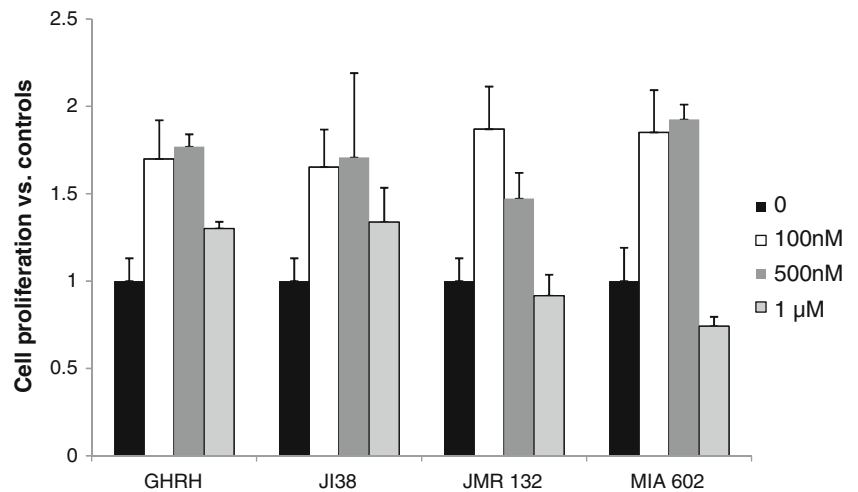


Fig. 3 Western blot analysis for SV1 expression in HaCaT keratinocytes cultured at low or high density and in the absence or presence of serum (FBS). Actin levels are shown as a loading control

Fig. 4 Proliferation of HaCaT keratinocytes in the presence of GHRH, agonist JI38, and antagonists JMR132 or MIA602 supplemented in the culture medium at 100 nM, 500 nM, or 1 μ M



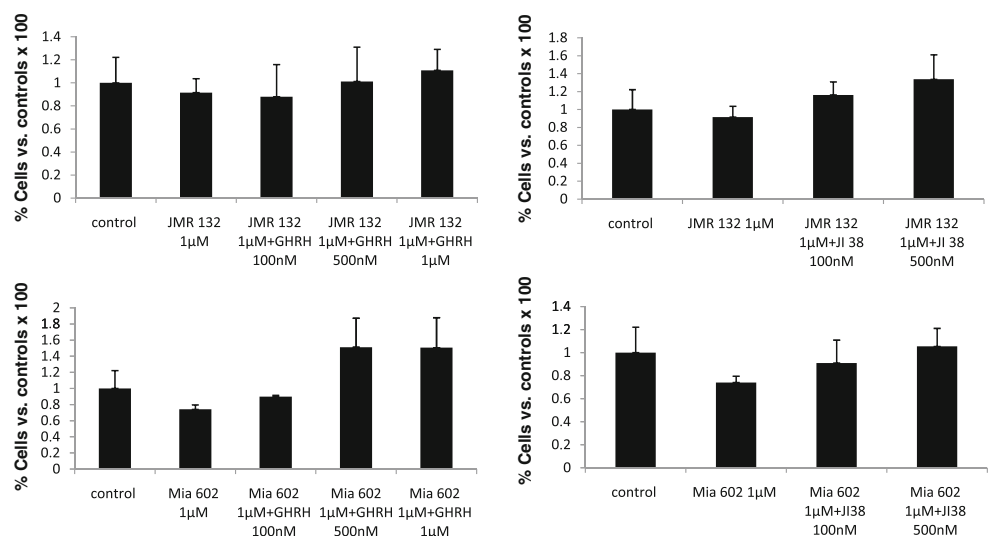
the immunohistochemical analysis, human pituitary tissue was used which showed positive staining for SV1 receptors (Fig. 2a). Omission of the primary antibody abolished the immunoreactivity against SV1 (Fig. 2b).

Effect of GHRH Analogs on the Proliferation and Migration of HaCaT Keratinocytes

In view of the increased anti-SV1 immunoreactivity in OSCCs, we then explored the effects of GHRH agonists and antagonists on the rate of proliferation of HaCaT keratinocytes in vitro. HaCaT cells are transformed keratinocytes of human origin that are not tumorigenic in vivo [44]. Therefore, we considered them as a reliable in vitro model for studies related to SCCs proliferation. Initially, we tested whether HaCaT cells express SV1. As shown in Fig. 3, western blot analysis indicated that, indeed, HaCaT cells are positive for SV1. We also noted

that SV1 expression was dependent on cell density: When cells were cultured at high density, SV1 expression was elevated as compared to that of cells growing at low-density conditions. In order to clarify whether this effect was due to contact inhibition at high density, we also explored the levels of SV1 expression in cells cultured at high or low density in the absence of serum. As shown in Fig. 3, upregulation of SV1 was detectable even in serum starved cells implying that the stage of cell cycle is not the determining factor in SV1 expression. Some inhibition though of SV1 in serum arrested, as compared to proliferating cells, cultured at low density was noted (Fig. 3). These observations are indicative of a dynamic role of cell contact in the regulation of SV1 expression. Subsequently, we tested whether HaCaT cells respond to GHRH analogs. We found that both GHRH and agonistic analog JI-38 as well as GHRH antagonists JMR-132 and MIA-602 were mitogenic at concentrations of 100 and 500 nM; however, at 1 μ M only GHRH and agonist JI-38

Fig. 5 Proliferation of HaCaT keratinocytes in the presence of antagonists JMR132 (upper panel) or MIA602 (lower panel) in combination with increasing concentrations of GHRH (left panel) or agonist JI38 (right panel)



stimulated cell growth while JMR-132 and the more potent MIA-602 inhibited it (Fig. 4). We then attempted to further explore the specificity of the GHRH antagonists used in this study. To that end, we tested whether increasing concentrations of GHRH and agonist JI-38 are capable of overcoming the inhibitory activities of JMR-132 and MIA-602 elicited at 1 μ M. Indeed, both GHRH and agonist JI-38 caused a dose-dependent increase of cell proliferation in HaCaT cells in the presence of JMR-132 and MIA-602 at 1 μ M (Fig. 5). Furthermore, while the agonists alone were highly mitogenic at 100 and 500 nM (Fig. 4), their mitogenic activity was diminished in the presence of the antagonists (Fig. 5).

The elevated SV1 expression under dense culture conditions prompted us to address whether GHRH analogs affect other properties of HaCaT cells besides cell proliferation, such as the rate of cell migration. Therefore, we performed an *in vitro* wound healing assay that is indicative of the ability of cells to migrate and is associated with the metastatic ability of the cancer cells. We chose to expose cells at 500 nM since at this concentration, all analogs exhibited mitogenic activity (Fig. 4), and thus, potential anti-migratory activity could be identified. As shown in Fig. 6, for GHRH, JI-38, and MIA-602, some inhibition in the rate of cell migration was detected which paralleled inversely the mitogenic activity of these analogs at equimolar concentration. However, JMR-132 at 500 nM potentially accelerated cell migration despite that at this concentration, it was less potent in terms of mitogenic activity as compared to the other three peptides tested. These results, besides their importance in the regulation of cell migration, indicate that regulation of cell proliferation and migration by GHRH analogs are not tightly related.

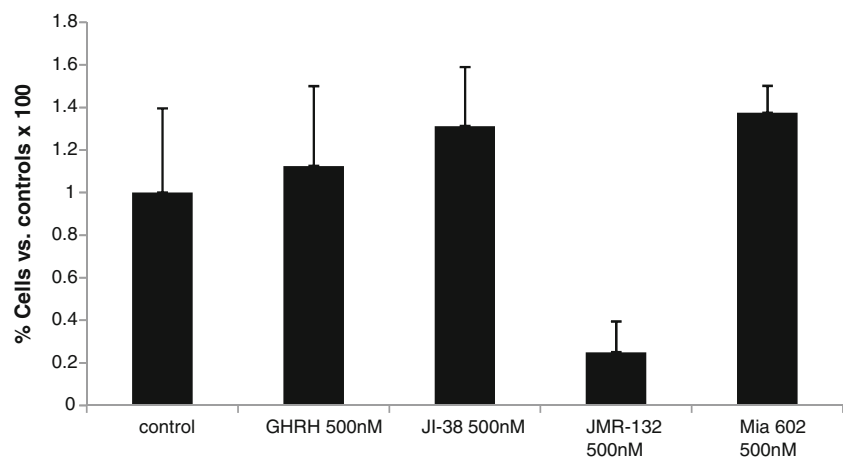
Discussion

Receptors for GHRH, particularly SV1, are expressed in several human cancers. Furthermore, antagonists of GHRH

are potent inhibitors of cancer cell growth *in vitro* and *in vivo* and have emerged as a promising therapeutic strategy for the treatment of several of these malignancies. In view of the absence of experimental results regarding the expression of GHRH receptors in oral neoplastic lesions, in the present study we evaluated the presence of SV1 immunoreactivity in OSCCs and dysplastic lesions by immunohistochemistry and in HaCaT keratinocytes by western blot analysis. Our results indicated that expression of SV1 in OSCCs is about five times more common than in the pre-malignant lesions (44 % in OSCCs vs. 9 % in precancerous lesions) suggesting that SV1 activity may be associated with malignant transition of oral squamous epithelium. This finding is in agreement with our previous findings on other cancers, such as melanomas, in which SV1 expression was significantly more common in the malignant than in the dysplastic lesions [36].

In view of the increased immunopositivity for SV1 in OSCCs, we subsequently explored the antineoplastic activity of GHRH antagonists *in vitro*, by using HaCaT human keratinocytes as a model. While HaCaT cells are immortal keratinocytes of skin origin, the fact that the oral epithelium also consists of keratinocytes justifies the use of these cells in order to assess the responsiveness to analogs of GHRH. We evaluated the effects of GHRH agonists and antagonists on two major phenotypic properties of these cancer cells such as the proliferation rate and the rate of cell migration. Our results confirmed that, indeed, GHRH antagonists inhibited the proliferation of HaCaT cells while GHRH and agonists stimulated it, at 1 μ M. We also noted that at lower concentrations, both agonists and antagonists were mitogenic. This stimulatory activity of GHRH antagonists at 100 and 500 nM has been noted by some of us (HK and AVS) using other antagonists in different cell lines. This might be associated with the sensitization of GHRH receptors such as SV1 by exposure at lower concentrations of GHRH analogs. This hypothesis that is consistent with the previously reported ligand-independent activity of SV1 [38]

Fig. 6 Cell migration (scratch) assay performed with HaCaT keratinocytes in the presence of GHRH, agonist JI38, and antagonists JMR132 or MIA602 supplemented in the culture medium at 500 nM



has to be confirmed by additional studies. The fact that GHRH and agonist JI-38 at increasing concentrations overcame the antiproliferative activity of GHRH antagonists in a dose-dependent manner argues in favor of the specificity of our findings. Despite that GHRH analogs clearly affected cell proliferation, cell migration was not affected considerably by GHRH, JI-38, and MIA-602. On the contrary, all these three analogs slightly inhibited the closure in vitro of the wound, despite that at the same concentration all three were mitogenic. GHRH antagonist JMR-132 though, which at 500 nM was less potently mitogenic as compared to the other three analogs, caused strong stimulation in cell migration. These discrepancies between the activities of the GHRH analogs used in the present study as regards on the effects on cell proliferation compared to migration indicate that these properties do not necessarily parallel each other. In addition, they may indicate that specific antagonists of GHRH may target additional receptors besides SV1 in the HaCaT keratinocytes and likely in other cells. These receptors upon their activation or downregulation differentially regulate that specific effect measured that is due to SV1 suppression. Finally, it may be due to the fact that cell migration and cell proliferation are occasionally opposing cellular activities, and thus, stimulation of cell proliferation may limit the capacity of these cells to migrate.

Collectively, our results demonstrate, for the first time, that OSCCs but not pre-malignant lesions such as dysplasias and hyperplasias frequently express the SV1 receptor for GHRH which suggests that this receptor splice variant might be associated with the progression of the disease. This finding, in addition with the responsiveness of transformed keratinocytes to GHRH analogs, indicates that antagonists for GHRH should be considered for the treatment of OSCCs.

Conflict of Interest The authors declare that they have no conflict of interest.

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