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Original Article

Effects of Over-Expression of LOC92912 Gene on Cell Cycle Progression

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Abstract

Background: We had previously identified the genes involved in squamous cell carcinoma of the head and neck using differential display and DNA microarray techniques. We also reported the first analytical study on a novel human gene called LOC92912, which was identified by differential display as a gene up-regulated in such carcinomas. LOC92912, which is a putative member of the E2 ubiquitin conjugating enzyme family, is located on chromosome 15q and encodes a protein of 375 amino acids. In this study, we present the extended analysis of LOC92912 gene in order to uncover the pathway implicated in cancer development or progression. We established a series of RPMI 2650 cell line permanently over-expressing LOC92912 gene, together with their related controls.

Methods: LOC92912 gene was cloned in pSG5-expressing vector. In vitro translation assay was performed using pSG5-expressing LOC92912. The construct was used for transient and permanent transfection of LOC92912 gene into RPMI 2650 cell line. Cell cycle analysis, clonogenicity, and cell growth assay for cells permanently over-expressing LOC92912 were performed. Focus-like formation studies, also, were investigated on cells permanently over-expressing LOC92912.

Results: We found that RPMI 2650 cells permanently over-expressing LOC92912 show an increase in the number of cells accumulated in G0/G1 phase of the cell cycle, a decrease in clonogenicity and cell growth and formation of focus-like structures. Preliminary data also showed changes in cell shape and cell-cell adhesion.

Conclusion: Our results demonstrated that LOC92912 induced alterations in the proliferation of cells and might represent a putative novel regulator of cell cycle and some other cellular functions. This novel human gene may also represent a new target for treatment of cancers.

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Keywords ● LOC92912 ● transfection ● clonogenicity ● cell cycle

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Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is an epithelial malignancy in oral cavity, larynx, oropharynx, and hypopharynx, which is the seventh most common cause of cancer-related death worldwide and represents about 90–95% of all malignant

neoplasms of the oral cavity.[1] Its incidence is rising rapidly all over the world,[2,3] as well as in developing countries,[4] during recent decades. The overall 5-year survival of patients with such carcinoma is one of the lowest among the major types of cancer and has not improved during the past decade despite advances in the diagnosis and management of the disease.[5,6] The disease is characterized by local tumor aggressiveness, early recurrence and high frequency of second primary tumors.[7] The risk factors for developing HNSCC include cigarette smoking,[8,9] alcohol consumption,[10,11] and human papilloma virus (HPV) infection.[12-14] Epstein Barr virus (EBV) infection is also associated with subtypes of nasopharyngeal carcinoma,[15] and there are evidences showing the implication of inheritance.[16] However, the pathogenesis of HNSCC includes a multi-step process that involves genetic and epigenetic changes of normal squamous mucosa to pre-malignant lesion and ultimately to overtly malignant tumors.[17] Many critical biological processes and pathways involved in HNSCC are still unidentified. One approach to uncover these pathways is to study novel genes with altered expression in related malignancies, in order to isolate elements involved in the genesis, progression, and metastasis of tumors.

In our previous studies on differential display analysis of HNSCC, a novel collection of cancer related genes was identified,[18] and some of those genes were studied in our laboratory.[19-22] The first analytical study of one of the novel genes, LOC92912, a hypothetical ubiquitin conjugating enzyme was reported earlier.[22] LOC92912 gene, encodes a protein of 375 amino acids containing a RWD domain, a coiled-coil and an E2 domain. We showed that LOC92912 mRNA was up-regulated in 85% of tumor samples of patients with HNSCC compared with the corresponding normal tissue. We also reported that the gene products were localized in the invasive epithelium and cancer islets of hypopharyngeal tumor samples. Using specific polyclonal antibody, we detected its exogenous and endogenous protein in a series of head and neck cell lines, although the expression level was relatively low compared with an internal control. We also purified the flag-tagged LOC92912 protein and found actin and six actin-related proteins as potential interacting partners, suggesting that LOC92912's functions may be linked to the cytoskeleton.

In the present report, we extended the analysis of this gene establishing a set of RPMI 2650 transfectants permanently over-expressing LOC92912.

Since carcinogenesis involves activation of oncogenes and/or inactivation of tumor suppressor genes, in the present study we further characterized this novel cancer related gene in order to uncover the pathway of its implication in cancer development or progression. Aiming to this goal we established a set of RPMI 2650 transfectants permanently over-expressing LOC92912. In this established cell line the effect of the over-expression of LOC92912 gene on cell cycle, cell growth, and clonogenicity was investigated. The formation of focus-like structure (foci) in cell culture as a consequence of the LOC92912 over-expression is also studied.

Materials and Methods

In Vitro Translation

In vitro translation assay was performed on pSG5-expressing LOC92912, using Promega's TNT Quick Coupled Transcription/Translation System. The whole procedure was performed according to manufacturer's instruction.

Generation of LOC92912 antibodies was done as described previously.[22]

Cell Culture, Expression Vectors, and Transfections

RPMI 2650 cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), at 37 ºC in a humidified atmosphere of 5% CO_2-95% air, as recommended by ATCC (American Type Cell Collection). Transfections were performed by calcium-phosphate method on RPMI 2650 cells.[23] Transfected cells were trypsinized 48 hours post-transfection and passaged into medium containing puromycin (2 µg/ml) to obtain permanent clones. The selection medium was replaced every 3 days and clones were isolated 20-24 days post-transfection. Typically, seven clones were isolated per permanent transfection and checked for over-expression of LOC92912 by Western-blotting. LOC92912 cDNA clone was obtained from ATCC (ATCC catalogue No.6394363; IMAGE clone ID: 3907760; Genebank I.D. BC017708; human uterus). The coding region was PCR-amplified using oligonucleotides containing BamHI restriction sites (CGCGGATCCCATTTAGCCATCTTCCTTTGGA GG and CGCGGATCCATGTCCGTGTCAGGG CTCAAG). The amplified products were cloned into the pSG5-puro-flag expression vector (IGBMC facility) to obtain sense and antisense versions of the recombinants.

Cell Cycle Analysis

To study the effect of LOC92912 on cell cycle progression, transiently and permanently

transfected cells with LOC92912 gene were analyzed by flow cytometry. For this purpose, 10^6 RPMI 2650 cells grown at sub-confluence were trypsinized, transferred to a tube containing medium with 10% serum and collected by centrifugation at 850 rpm. The pellets were washed with phosphate buffered saline (PBS), re-suspended in 100 µl PBS, followed by adding 3 ml ethanol 70% (-20°C) and incubated for 1 hour at 4°C. Cells were then washed twice with PBS followed by the addition of staining solution containing RNase A (100 µl of 10 µg/ml) and propidium iodide (200 µl of 100 µg/ml). Detached cell populations were also collected and combined with adherent cells for all experiments. The cell suspensions were passed through the Sefar AG Filtration Division (pore size: 80 µm) or filtration syringe of 50 µm pore size (BD BIOSCIENCE, USA) to remove aggregates. Then 20,000 cells were analysed using Fluorescence-activated cell sorting, FACS caliber cytometer (Becton Dickinson). The doublets as well as debris were excluded from the analysis. Cellquest software was used for acquisition and Modfit software (Verity) for cell cycle analysis.

To conduct the same experiment on the cells transiently transfected with pSG5-LOC92912, exponentially growing cells were co-transfected with 18 µg of the pSG5-LOC92912 and 2 µg of pCMVCD20 (as explained in transfection section). Forty-eight hours after transfection detached cell populations in combination with attached cells were collected and labeled with 10 µl of fluorescein isothiocyanate (FITC)-conjugated anti-CD20 antibody for 30 min. Cells were then washed twice with PBS and after fixation, were stained with propidium iodide. About 20,000 CD20-positive cells were routinely analyzed for their DNA content. The doublets as well as debris were eliminated by appropriate gating.

Cell Growth / Survival Assays

A colorimetric method based on MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide), (CHEMICON International, Inc., USA) was used for *in vitro* growth assays and the proliferation of RPMI 2650 cells were evaluated over 20 days. The growth pattern of RPMI 2650 over-expressing LOC92912 was compared with controls. Different RPMI 2650 permanent transfectants (five independent clones of each recombinant including sense and antisense of LOC92912-pSG5 and empty vector) were plated at a density of 4000 cells/wells in 96-well culture plates, each one in triplicate. The final volume of cell culture medium was 100 µl, supplemented with 2 µg/ml puromycin. The medium was changed regularly, every three days, and plated corresponding to different time points including: 1, 4, 7, 10, 13, 16, 18, and 20 days and then the cells were analysed by MTT assay according to the manufacturer's instruction. The absorbance of lysates was measured with an ELISA plate reader at the wavelength of 570 nm. The growth curve was represented by the average absorbance of each stable transfectant preparations compared with that of control cells. The data were subjected to Student's *t* test and standard deviation of each point was calculated.

Clonogenicity Assay

To study the effect of LOC92912 in colony formation, clonogenicity assay was carried out using permanently transfected RPMI 2650 cells with a plasmid expressing sense LOC92912 (pSG5-LOC92912), or the control vector (pSG5-antisense LOC92912 and empty vector). All the experiments as well as the plasmid preparations were done in triplicate. After 2 or 3 weeks of incubation with regular changes of growth medium containing 2 µg/ml puromycin (Invitrogen), the cells were washed twice with ice-cold 1x PBS and fixed with methanol 100% (stored at -20ºC) for 10 minutes. Colonies were visualized by crystal violet and photographs were taken with a Nikon COOLPIX 775 camera.

Foci Formation

$4x10^6$ cells of individual clones (5 different independent clones of each permanent transfectant) were seeded in 100-mm dishes and grown for 5-6 days. The normal cell growth of controls and foci formation in sense-LOC92912 cells was recorded by using a LEICA DM IRB microscope with objective lens magnification of 10x.

Results

In Vitro Translation Assay

We have previously reported the expression of LOC92912 in different head and neck cell lines.[22] *In vitro* translation assay was performed to determine the protein product of the LOC92912 gene, cloned in pSG5 mammalian expressing vector. Results presented in figure 1 clearly show that LOC92912 gene encodes a protein product of about ~ 44kDa in size.

Cell Cycle Analysis of Permanent Transfectants

A set of permanent transfectants were obtained from RPMI 2650 cell line. The DNA content of individual clones was determined by flow cytometry. As shown in figure 2, LOC92912 over-expression resulted in an accumulation of cells in G0/G1. We found about

20-25% increase in the G0/G1 fraction in RPMI cells over-expressing LOC92912 compared with the controls consisting of parental cells and cells transfected with empty vector or antisense-LOC92912. This increase was accompanied by an average reduction of about 8% in the cells in S phase and a decrease of 10-20% of cells in G2/M. These results were observed in five different clones of each permanent transfectant and the related transiently transfected RPMI cells. Thus, the expression of LOC92912 may inhibit cell cycle progression of permanently or transiently transfected human head and neck cancer cell line, RPMI2650.

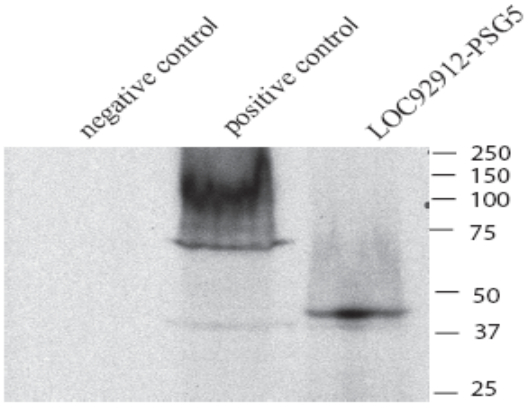

Figure 1: In vitro translation of the vector encoding LOC92912. The protein derived from the expression vector has the expected molecular size. Lane 1: negative control (no template); lane 2: positive control (luciferase gene); lane 3: LOC92912 expression vector (sense-LOC92912-pSG5). Molecular markers are displayed on the right.

Cell Survival Analysis

The in vitro growth assay revealed that the up-regulation of LOC92912 diminishes the growth rate by 30%, 20%, and 10% at the seventh, tenth, and thirteenth days after seeding, respectively (figure 3). This suggests that over-expression of LOC92912 inhibited the growth rate of cells *in vitro* and compromised their survival in parallel with the abnormal DNA pattern found in cells over-expressing LOC92912.

Clonogenicity Assay

As shown in figure 4, the transfection of pSG5-LOC92912 in RPMI cells markedly decreased the number of colonies compared with the controls [cells transfected with empty vector (pSG5) or the complementary strand of LOC92912 (pSG5-antisense)]. These results suggested that the over-expression of LOC92912 may inhibit cell growth and colony formation potential.

Focus-like Formation Phenotype

As shown in figure 5, RPMI 2650 permanently over-expressing LOC92912 formed characteristic foci-like structure in culture. This particular feature prevented these cells to grow normally and to reach confluence, whereas the cells transfected with empty vector, antisense-LOC92912 and parental cells were growing satisfactorily under the same standard conditions. This feature was highly frequent in cells over-expressing LOC92912 and was absent in controls.

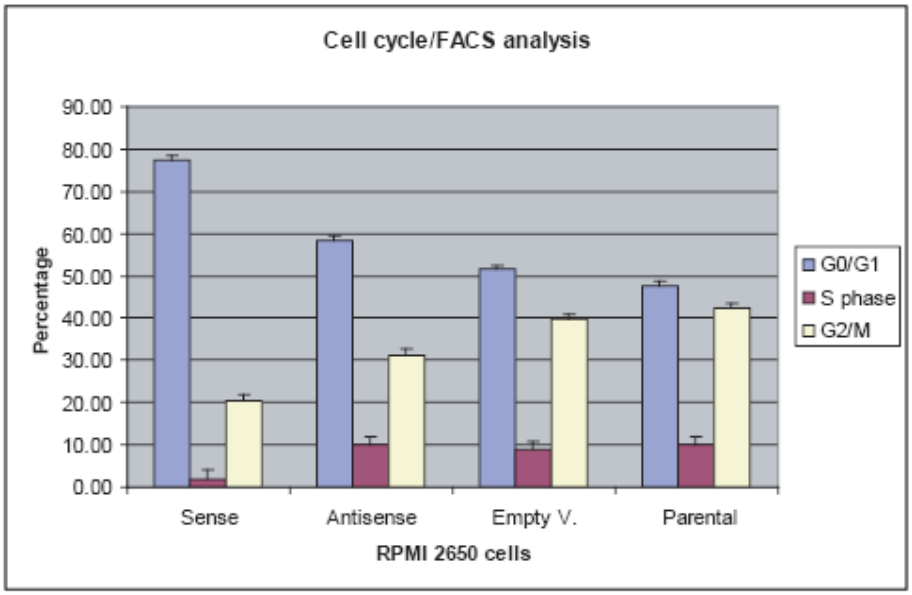

Figure 2: Cell cycle analysis of the RPMI2650 permanent clones. Cells over-expressing LOC92912 show higher levels of cells in the G0/G1 fraction compared with the controls and smaller ratio of cells in S phase.
Sense: RPMI2650 cells permanently over-expressing LOC92912, Antisense: RPMI2650 cells permanently transfected with the antisense version of LOC92912, Empty vector: RPMI2650 cells permanently transfected with empty vector, parental: parental RPMI2650 cells.

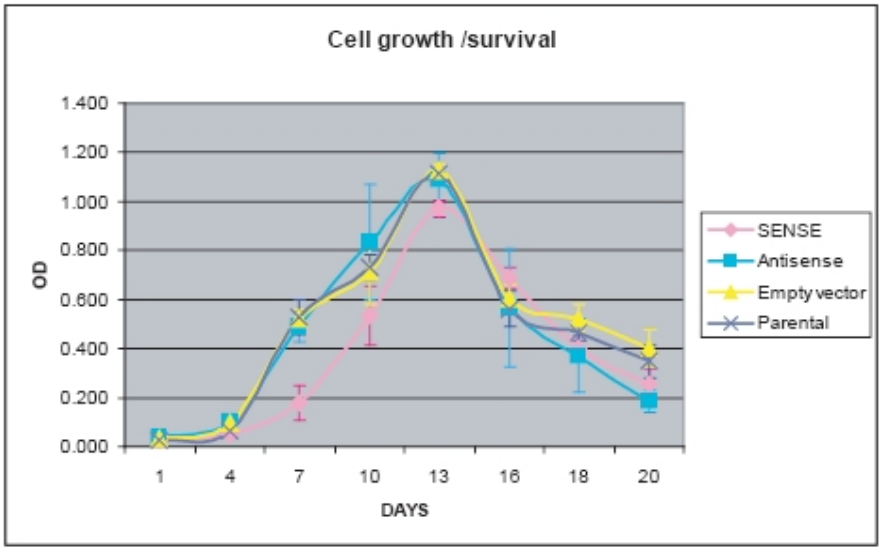

Figure 3: Effect of LOC92912 over-expression on the growth of RPMI2650 cells. The viability of the permanent transfectants were analyzed in triplicate for 5 independent clones of each permanent trasnfectant and control cells (sense, antisense and empty vector), using MTT assay. Sense, antisense, and empty vector indicate RPMI cells permanently transfected with the sense version of LOC92912, the antisense version and empty vector, respectively. Parental: parental RPMI2650 cells. The effect of LOC92912 expression on the exponential growth of cells from day 7 to 13 is seen.

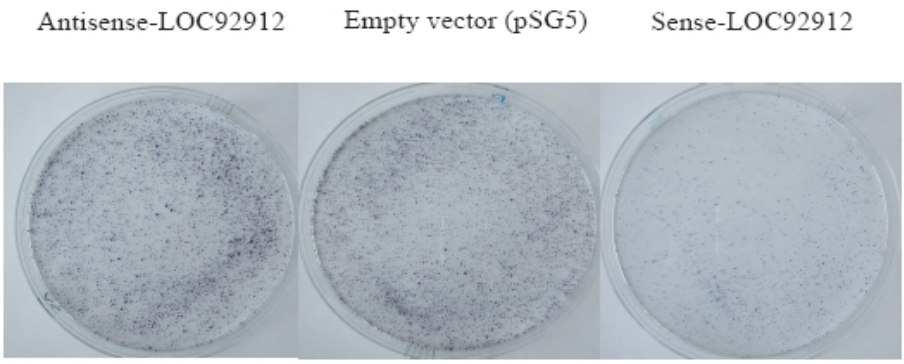

Figure 4: Clonogenicity assay of LOC92912 in RPMI2650 cells. Cultured dishes display the distribution of colonies and variation in the number of colonies after 15 days of culture in different plates. The significant differences in the number of colonies between cells over-expressing LOC92912, cells transfected with antisense-LOC92912, and control cells transfected with empty vector (pSG5) are obviously visible. This observation was confirmed by three independent experiments each one in triplicate, using different batches of the cells or maxi preparation of plasmids (LOC92912-pSG5, antisense- LOC92912-pSG5, and empty vector; pSG5).

Discussion

The present study provides the second descriptive analysis of the novel human gene LOC92912 that was constructed as pSG5-puromycine-flagged N-terminal-LOC92912 and reported earlier.[22] This gene which is up-regulated in hypopharyngeal tumors was identified by differential display analysis in our laboratory,[18] and characterized partially in above mentioned report. We found that actin and 6 actin-associated proteins co-purify ambiguously with this protein, which could represent its potential interacting partners.

In the present study, we established a set of RPMI 2650 transfectants permanently over-expressing LOC92912 to perform a biased approach to cancer molecular biology, assuming that the molecular processes determined by an oncogene or tumor suppressor gene in cultured cells, can reflect the most of the in vivo events that lead to cancer. We aimed to identify the signatures that can be subsequently validated for implication of LOC92912 gene in cancer as well as providing a better understanding about pathways that are interrupted in cancer.

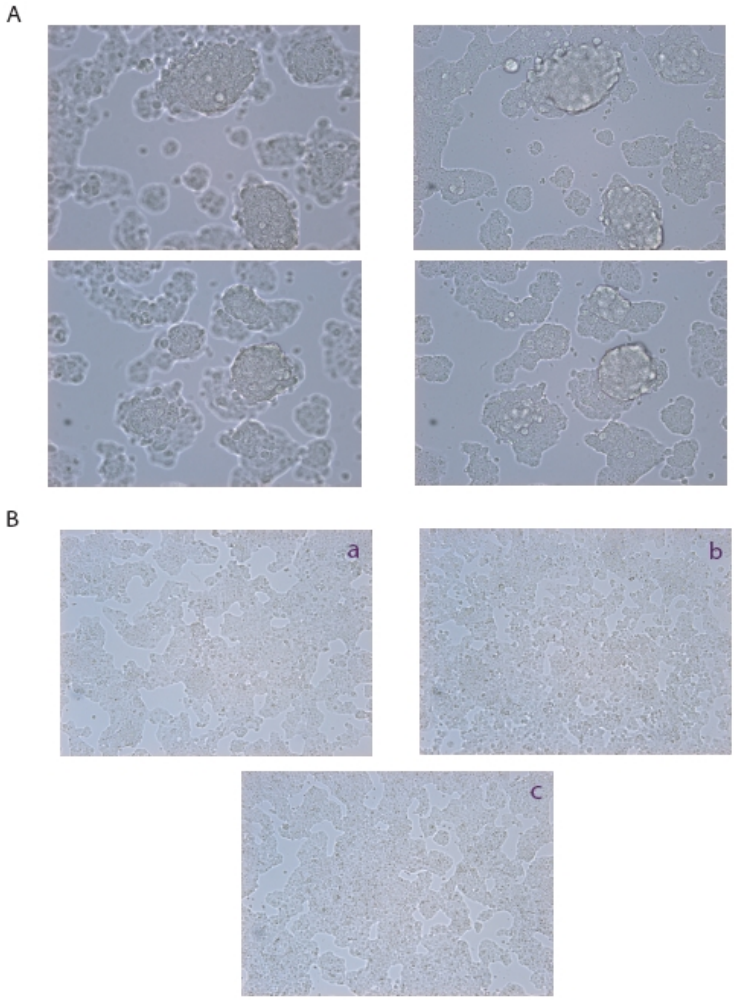

Figure 5: The permanent over-expression of LOC92912 induces the formation of foci. A: RPMI2650 cells over-expressing LOC92915. Note the apparition of cells growing vertically in colonies. This particular phenotype prevents cells to reach confluence as in controls. Photos on the left are focused on the top of the foci; the photos on the right show the same field focusing the background cells. B: Control cells consisting of parental cells (a), cells transfected with empty vector (b), and cells harboring the antisense version of LOC92912 (c). Photos were taken under phase contrast (objective lens magnification: 10x).

In this over-expressing system, some normal functions of the cells were altered when compared with the controls including parental cells or cells permanently transfected with empty vector or antisense-LOC92912. These altered parameters include increased number of cells accumulated in G0/G1 phase in cell cycle, decreased clonogenicity and cell growth/survival and the apparition of aggregate structures or foci as result of vertical growth only in cells over-expressing LOC92912. Preliminary data also showed an alteration in cell shape and cell-to-cell adhesion and attachment (data not shown).

Regulation of cell proliferation depends on numerous internal and external factors, among them the integration of signal transduction systems that are activated by external signal molecules, such as growth factors and extra cellular matrix components are of high importance.[24] Dependent on these signal transduction networks, the cell decides, in the G1 phase, to continue proliferation or, alternatively, to stop cell-cycle progression, and undergo apoptosis, differentiation, or quiescence.[25] Among all the factors affecting cell cycle, actin, which was identified as a potential interacting partner for LOC92912,[22] has been demonstrated to play an important role in the regulation of G1-phase progression.[26-30] Actin, one of the most important proteins of cytoskeleton, is essential in the process of cytokinesis and cell spreading and is involved in G1-phase progression. In addition, as an intermediate factor in signal transduction, actin is likely to be involved in cell-cycle regulation induced by external signal

molecules. It seems that our observations regarding the formation of foci or aggregates of the vertically growing cells, in cells over-expressing LOC92912 may indicate that these cells have altered cellular divisions.

On the other hand, it has been reported that the overexpression of drebrin, one of the actin-associated protein co-purified with LOC92912 in cultured cells,[22] results in the formation of dendrite-like cell processes.[31] This phenotype (cell shape) was also observed in about one forth (3 out of 12) of the clones overexpressing-LOC92912 gene. Furthermore, one in twelve clones of cells permanently over-expressing antisense-LOC92912 showed a size of about five times more than non-transfected parental cells (data not presented). While not much is known about the subsequent steps that lead to remodeling of actin cytoskeleton and concomitant cell shape changes, the role of this protein and its associates should be considered. However the association of our gene of interest with the structural and functional alterations of actin cytoskeleton or actin related proteins (i.e. Gelsolin and drebrin) as well as the implication of this gene in cell cycle and division, considering its hypothetic role as ubiquitin conjugating enzyme, needs to be further investigated.

Over-expression of LOC92912 gene also resulted in decreasing cell growth and clonogenicity.

The data seem to be in the conflict with our previous report that showed up-regulation of this gene in the tumor samples. While such a contradiction has been reported for other proteins as well,[32] there may be several reasons for this conflict:

A; it has been shown that the transcript was over-expressed by an average of 2.4-fold in tumor samples compared with the normal,[22] while in the cells permanently over-expressing LOC92912, the over-expression of this gene may be tens of times more compared with *in vivo* situation and it may cause this contradiction. Therefore, whereas the *in vitro* role of the gene over-expression, in suppressing cell growth and cell cycle progression, suggests its role in tumor suppression, further *in vivo* study is needed to confirm this hypothesis.

B; it is possible that while the protein is over-expressed in the tumor cells, it can not possess its normal function.

C; While the experiments with cultured cells are fast and cost effective, the tumor cells have genetically adapted to the culture conditions and the *in vitro* systems are devoid of tumor-host interactions. Therefore the extrapolation of the *in vitro* results to the human situation is very difficult. The development of a (mouse) model for HNSCC that closely mimics the human situation would be beneficial to uncover the role of this gene and to detect new key genes in HNSCC genesis. This information may be served as a tool for designing new treatment modalities. However finding a link between this gene and carcinogenicity (*in vivo* assay in knock out mice), and its successful use in cancer prognosis, diagnosis, and/or treatment is a long term perspective for future work.

It would be also interesting to study the potential substrates for this ubiquitin conjugating enzyme. The ubiquitin–proteasome pathway and its upstream system are responsible for 80% of the cell's protein degradation, and thereby have a major role in cellular homeostasis. The proteins of all vital compartments including cytoplasm, nucleus, and membrane are kept under its regulatory control. The rewards of developing agents that can specifically interfere with the E2/ E3 system and their interaction with the substrates, will remarkably be high in the area of cancer treatment. Thus, the understanding of the complex ubiquitination system and its explicit interactions and mechanisms may lead to more innovative cancer therapies, which will offer the hope for better and perhaps greater curative successes in malignant and other diseases.

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Conflict of Interest: None declared

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