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Original Article

ISG20 is overexpressed in clinically relevant radioresistant oral cancer cells

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Abstract: Global standard fractionated radiotherapy (RT) for the treatment of malignancies consists of X-ray irradiation with 2-Gy/day, 5 days a week for 5-7 weeks. Recently, clinically relevant radioresistant (CRR) cells were first defined as cells that can continue to grow even after exposure to daily 2-Gy of X-rays for more than 30 days in vitro. To analyze the characteristics of radioresistant cancer cells, CRR oral cancer cells (CRR-OCCs) were established, and the expression level of interferon-stimulated exonuclease gene 20 (ISG20) was evaluated with qRT-PCR and immunohistochemical analysis. Our result revealed that the expression level of both ISG20 mRNA and its protein in CRR-OCCs were higher than those of corresponding parental cells. We concluded that ISG20 was statistically overexpressed in CRR-OCCs. ISG20 overexpression may be necessary for the radioresistant phenotype in CRR-OCCs, and targeting ISG20 of human cancer cells may lead to more efficient RT or chemoradiotherapy for eliminating cancer.

Keywords: Clinically relevant radioresistance, oral cancer cells, fractionated radiotherapy, ISG20

Introduction

Radiotherapy (RT) and RT combined with concurrent systemic chemotherapy have remained major treatment modalities in patients with head and neck cancer (HNC) including oral cancer [1]. Postoperative RT is also a standard treatment in HNC cases with established high risk factors such as incomplete resection margins or lymph node metastasis with extracapsular spread (ECS) [2]. Nowadays, particle beam therapy (PT), particularly proton beam therapy (PBT) or carbon-ion radiotherapy (CIRT), has also been selected because of high dose conformity with an advantageous biologic effect in comparison to photon therapy, and superior dose distribution with reduced dose to the normal tissue compared to RT [3, 4].

Radioresistance of malignancies has remained a critical obstacle in RT or chemoradiotherapy (CRT). Currently, for example, one course of intensive modulated RT (IMRT) for cancer treatment is composed of fractionated radiation (FR) with approximately 2 Gy of X-ray irradiation/day, for 5 days a week, over a 5-7 week period [5]. Kuwahara and Fukumoto et al. have first defined CRR cells as the cells that can continue to grow even after exposure to 2 Gy/day of X-rays for more than 30 days (total dose >60 Gy) [6]. In order to develop more effective radiotherapies against radioresistance, they established human novel CRR cell lines one of which was CRR oral cancer cells (CRR-OCCs), SAS-R1 (6-10). These cells continue to proliferate and grow under the condition of exposure to 2 Gy/day for more than 30 days in vitro. Total RT dose to these CRR cells over the whole process has added up to more than 1500 Gy. According to the results of cDNA microarray analyses for differential gene expression in association with the CRR phenotype, they revealed that two genes, the guanine nucleotide-binding protein 1 (GBP1) and the interferon stimulated exonuclease gene 20 (ISG20), were overexpressed in SAS-R1 [7]. Among them, GBP1 gene expression was higher in CRR cells

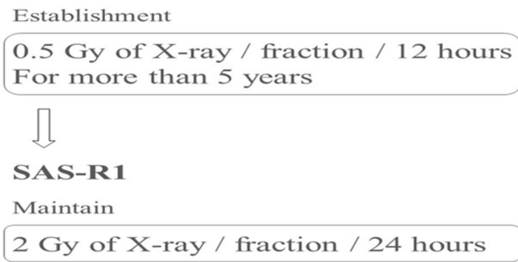

Figure 1. Schematic illustration of establishing CRR cell line SAS-R1. SAS-R1 was established by exposing SAS cells to 0.5 Gy of X-rays at every 12 hour intervals for more than 5 years. For maintenance of the CRR phenotype, 2 Gy of X-rays was carried out every 24 hours.

than in their corresponding parental cells. They also found that GBP1 knockdown by siRNA reversed radioresistance of CRR cells in vitro and in xenotransplanted tumor tissue. Furthermore, according to the results of immunohistochemical analysis for clinical relevance of GBP1 expression in patients with HNC, cases that were GBP1-positive predicted inferior response to RT. They finally concluded that GBP1 overexpression is necessary for the radioresistant phenotype in CRR cells [7].

On the other hand, the expression level of ISG20 in CRR cells and the relationships between ISG20 levels and the radioresistant phenotype has been unclear. ISG20 was identified from an oligo microarray [11], and plays an important role in immune response against various infections including oncogenic virus such as hepatitis viruses or Kaposi sarcoma-associated herpesvirus (KSHV) [12, 13]. It has recently been reported that overexpression of ISG20 induced by thyroid hormone in hepatocellular carcinoma (HCC) has led to significant progression of metastasis and angiogenesis, and that higher ISG20 expression was significantly correlated with poorer recurrence-free survival [14]. Another group has showed that ISG20 was overexpressed depending on the progression of cirrhosis, in patients with LC plus HCC. In patients with HBV-related HCC, ISG20 levels were also induced by HBV infection and significantly associated with progression and clinical outcome [15]. These two groups indicated ISG20 overexpression has an oncogenic role for tumor progression and might be a predictor for clinical outcome in a subset of HCC [14, 15].

In this study, our result showed that the expression of both ISG20 mRNA and its protein in CRR-OCCs were higher than those of corresponding parental cells. We concluded that ISG20 overexpression may be necessary for the radioresistant phenotype in CRR cancer cells as well as the relationship between GBP1 overexpression and radioresistance.

Materials and methods

Cell lines and cell culture

Human oral cancer cell line SAS was obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). CRR-OCCs, SAS-R1, was established by exposing these parental cells to irradiation at 0.5 Gy of X-rays every 12 hours for more than 5 years [3]. For maintaining the CRR phenotype, FR at 2 Gy/day was performed. These cells were maintained in RPMI-1640 medium (Nacalai, Kyoto, Japan) with 5% FBS (Invitrogen, CA, USA) in a humidified atmosphere in air with 5% CO_2 at 37°C.

Irradiation

Irradiation of X-rays at a dose rate of 1.0 Gy/min was performed in a 150-KVp X-ray generator (MBR-1520R; Hitachi, Tokyo, Japan) with a total filtration of aluminum 0.5 mm plus copper 0.1 mm filter (**Figure 1**).

Quantitative reverse transcription-PCR

Total RNA of SAS and SAS-R1 was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized by RT using Rever Tra Ace qPCR RT Master Mix (TOYOBO). The qRT-PCR of ISG20 was carried out using the primer set 5'-CGACACGTCCACTGACAGGC-TGTTG-3' and 5'-TCCATCGTTGCCCTCGCATCT-TC-3' [12]. The qRT-PCR was performed using RT^2 SYBR Green ROX qPCR Mastermix (QIAGEN). The PCR condition was: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 58°C for 30 s and 72°C for 30 s using the Mx3005P qPCR System (Agilent).

Antibodies

The primary antibody used was anti-ISG20 (GTX114499; GeneTex). The secondary anti-

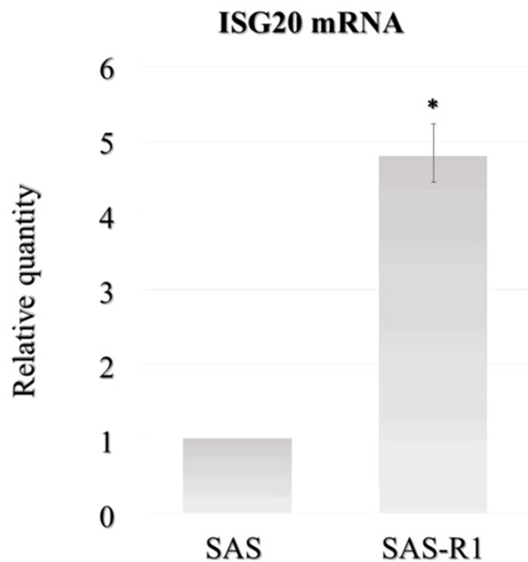

Figure 2. Overexpression of ISG20 gene in CRR-OCCs. The relative levels of ISG20 mRNA in SAS-R1 cells was higher than that of corresponding parental cells by qRT-PCR. *P<0.001.

body used was goat anti-rabbit IgG (H1202; Nichirei).

Animal experiments

This study was carried out as described previously [8, 16].

Immunohistochemistry

Tumor tissue was fixed in 10% formalin. For immunohistochemistry (IHC), sections were deparaffined with xylene, and rehydrated through an ethanol series and PBS. Antigen retrieval was performed by heat treatment by microwave for 20 min with citrate buffer, pH6.0. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min, followed by incubation with G-Block (Genostaff) and avidin/biotin blocking kit (Vector). The sections were incubated with anti-ISG20 rabbit antibody at 4 degrees Celsius overnight. They were incubated with biotin-conjugated goat anti-rabbit Ig (Dako) for 30 min at room temperature followed by the addition of peroxidase conjugated streptavidin (Nichirei) for 5 min. Peroxidase activity was visualized by diaminobenzidine. The sections were counterstained with Mayer's hematoxylin (MUTO), dehydrated, and then mounted with Malinol (MUTO).

Statistical analysis

Experiments in vitro were done in duplicate. Data were expressed as means ± S.D., and were statistically analyzed using Student t-test with StatMate V software. A *P*-value of <0.05 was considered significant.

Results

Expression level of ISG20 mRNA

To evaluate the expression pattern of ISG20 in CRR-OCCs, we analyzed the mRNA expression by qRT-PCR. The expression level of ISG20 mRNA was increased in SAS-R1 cells at 4.8 times greater than that of their corresponding parental cells (**Figure 2**).

Immunohistochemistry of ISG20 in xenotransplanted tumor tissues

SAS-R1 tumors transplanted into nude mice are also resistant to FR [16]. The ISG20 polyclonal antibody showed distinct cellular staining in tumor cells. Immunohistochemical staining patterns and distribution revealed a diffuse cytoplasmic staining and intensive nuclear localization (**Figure 3**). Transplanted SAS tumor cells showed weak but detectable ISG20 staining in the cytoplasm and nucleus (**Figure 3A**). In contrast, SAS-R1 tumor cells were moderately to strongly positive for ISG20 staining in both of cytoplasm and nucleus. Particularly, the cytoplasmic staining in SAS-R1 cells was more intensive than that of SAS cells (**Figure 3B** and **3C**), while surrounding normal connective tissue, blood vessels, or stromal cells were not stained with anti-ISG20 antibody.

Discussion

RT and CRT with FR remain global standard treatment modalities in human malignancies [1, 2]. Recently, because of its superior dose distribution and advantageous biological effect, PT such as particle beam therapy (PBT) and carbon-ion radiotherapy (CIRT) has been selected for the treatment of many kinds of malignant tumors [3, 4]. However, in spite of the development and progression of RT, it has been very hard to conquer radioresistance of malignancies.

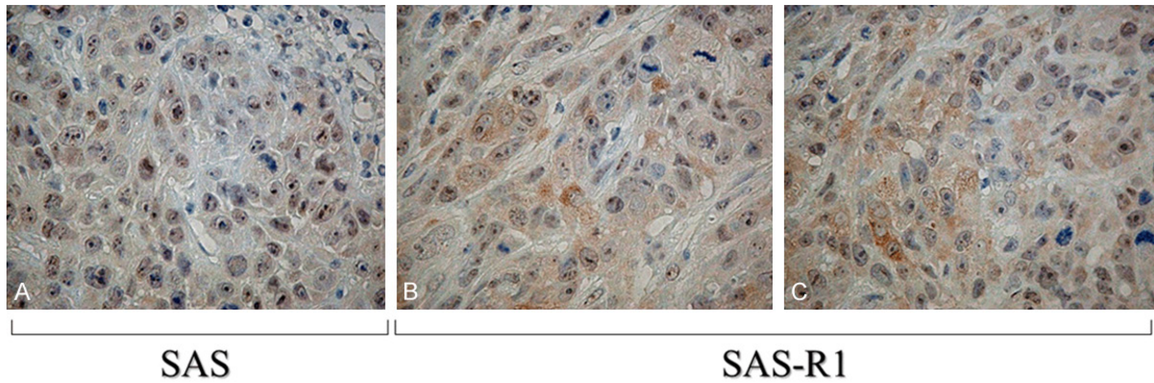

Figure 3. Immunohistochemical staining patterns of ISG20 protein in tumor tissues xenotransplanted into nude mice. The distribution revealed diffuse cytoplasmic staining and intense nuclear localization. A. SAS cells treated with anti-ISG20 antibody show weak staining in cytoplasm and nucleus. B. SAS-R1 cells show moderate ISG20 staining. C. Intense ISG20 staining is observed in tumor cells. The cytoplasmic staining in SAS-R1 cells was high compared to that of SAS cells. Surrounding normal connective tissue, blood vessels, or stromal cells are not stained with anti-ISG20 antibody.

Kuwahara and Fukumoto et al. previously reported that they had defined CRR cells as cells that can continue to grow after X-rays irradiation of 2 Gy/day for more than 30 days, with total dose >60 Gy [6]. They established CRR cancer cell lines SAS-R1, HepG2-8960-R, and KB-R from three kinds of human cell lines: SAS, HepG2, and KB by exposing these parental cells to FR of X-rays for more than 5 years, respectively [8, 9]. In microarray analyses, they found that two candidate genes, GBP1 and ISG20, were overexpressed and were expected to be responsible for the radioresistant phenotype of CRR cancer cells. They clarified that GBP1 was overexpressed in CRR cancer cells and the translational level of GBP1 was closely correlated to the transcriptional step. According to these findings, they finally indicated that GBP1 overexpression is associated with radioresistance, and it is mainly regulated at the transcriptional step. Moreover, they reported that knockdown of GBP1 reduced radioresistance to the level of parental cells in all the CRR cells, and this radiosensitization by GBP1 knockdown was also observed in transplanted tumors to nude mice. However, although GBP1 was transfected to parental cells, they did not reveal significant changes of radioresistance. Only GBP1 knockdown in CRR cells induced apoptosis through increased radiation-induced DNA double strand breaks (DSBs). GBP1 knockdown enhanced radiation induced early apoptosis through the delay of DSB repair in CRR cells. These results suggest that although GBP1 is essential to deal with radiation, it is not enough to shift the cell into CRR. They also indicated that cases with GBP1-positivity predicted inferior response to RT in patients with head and neck malignancies including oral cancer [8]. Sublethal damage that is induced by FR is suggested to be restored by homologous recombination repair (HRR) of DSBs [17]. Because parental cells with GBP1 overexpression do not reveal radioresistance, this suggests that HRR requires multiple molecules other than GBP1.

On the other hand, further analysis for evaluating the expression level of ISG20 in CRR cells has not been performed, and the relationship between ISG20 levels and the radioresistant phenotype has been controversial. In this study, we detected ISG20 gene, and evaluated the expression level in CRR cells and analyzed the relationships between ISG20 levels and radioresistance of CRR cells.

ISG20, which is located on human chromosome 15q26, is an RNA exonuclease that can cleave single-stranded RNA and DNA [18, 19]. ISG20 expression is induced by both type I (IFN-α/β) and type II (IFN-γ) IFNs in various kinds of cell lines, and its antiviral activity has been induced by NF-κB and IRF1 activation [20]. Thus, ISG20 plays an important role in mediating interferon's antiviral activities. With laser confocal microscopy, ISG20 protein is primarily detected in the nucleus with only a small amount in the cytoplasm [21]. Gongora

et al. reported that ISG20 was distributed diffusely throughout the nucleoplasm in 30% of positive CCL13 cells. They strongly suggested that progression of the cell cycle altered the distribution of ISG20 in the intranuclear compartment [22]. It is also reported that ISG20 has a role in controlling cellular proliferation and differentiation by mediating estrogen [23].

Kaposi sarcoma (KS) is the most common HIV/AIDS-associated tumor, and it is caused by the Kaposi sarcoma-associated herpesvirus (KSHV). In patients with KS, involvement of the oral cavity portends a poor prognosis [24, 25]. Dai et al. found that interferon-stimulated genes were highly upregulated in KSHV-infected oral fibroblasts. Particularly, ISG15 and ISG20 are required for maintenance of virus latency through regulation of specific KSHV microRNAs. According to these findings, they demonstrated that the maintenance of latency of oncogenic virus is closely related to the overexpression of interferon-stimulated genes such as ISG20 [13].

Lin has reported that ISG20 expression was induced by thyroid hormone and the forced expression of ISG20 led to significant promotion of metastasis and angiogenesis in HCC [14]. They also found that ISG20 overexpression in patients with HCC was positively correlated with clinical factors such as vascular invasion and tumor size, and higher ISG20 expression was significantly correlated with poorer recurrence-free survival. They concluded that higher expression of ISG20 induced by thyroid hormone has an oncogenic role for tumor progression in a subset of HCCs. Van Tong found that ISG20 was overexpressed according to the progression of cirrhosis in patients with liver cirrhosis (LC) plus HCC, and ISG20 levels were significantly associated with progression and clinical outcome in patients with HBV-related HCC [15]. Thus, ISG20 might be an indicator for liver injury and clinical outcome in HBV-related HCC.

In this study, we confirmed that the expression level of ISG20 in CRR-OCCs was higher than that of corresponding parental cells by qRT-PCR and immunohistochemical staining. The staining pattern revealed that ISG20 protein was primarily detected in the nucleus with diffuse cytoplasmic staining in cancer cells. Furthermore, the cytoplasmic staining in SAS-R1 cells was higher than that of SAS cells. Gongora's report supports these results [22].

We conclude that ISG20 overexpression may also be necessary for the radioresistant phenotype in CRR cancer cells as well as the relationship between GBP1 overexpression and radioresistance. Targeting ISG20-positive cancer cells will be an efficient method in conquering cancer. Further studies are required such as analyzing the relationships between ISG20 knockdown and radiosensitization in CRR-OCCs, and evaluating the correlation between ISG20 overexpression and radioresistance or prognosis in patients with oral cancer treated with RT. We plan to investigate whether ISG20 levels can be a predictive marker for radioresistance in human malignancies including oral cancer treated with PT such as PBT or CIRT.

Conclusion

Expression level of ISG20 in CRR-OCCs was higher than that of their corresponding parental cells. ISG20 overexpression may also be necessary for the radioresistant phenotype in CRR cancer cells.

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Disclosure of conflict of interest

None.

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References

[1] Gupta T, Kannan S, Ghosh-Laskar S and Agarwal JP. Systematic review and meta analyses of intensity-modulated radiation therapy versus conventional two-dimensional and/or or three-dimensional radiotherapy in curative-intent management of head and neck squamous cell carcinoma. PLoS One 2018; 13: e0200137.

[2] Maihoefer C, Schüttrumpf L, Macht C, Pflugradt U, Hess J, Schneider L, Woischke C, Walch A, Baumeister P, Kirchner T, Zitzelsberger H, Belka C and Ganswindt U. Postoperative (chemo) radiation in patients with squamous cell cancers of the head and neck - clinical results from the cohort of the clinical cooperation group "personalized radiotherapy in head and neck cancer". Radiat Oncol 2018; 13: 123.

[3] Cubillos-Mesías M, Baumann M, Troost EGC, Lohaus F, Löck S, Richter C and Stützer K. Impact of robust treatment planning on single- and multi-field optimized plans for proton beam therapy of unilateral head and neck target volumes. Radiat Oncol 2017; 12: 190.

[4] Fuwa N. The present and future status of particle therapy in head and neck cancer. J Jpn Soc Oral Tumor 2016; 28: 128-33.

[5] Tannock I. The Basic Science of Oncology, 4th edition. New York NY McGraw-Hill: Medical Publications; 2005.

[6] Kuwahara Y, Li L, Baba T, Nakagawa H, Shimura T, Yamamoto Y, Ohkubo Y and Fukumoto M. Clinically relevant radioresistant cells efficiently repair DNA double strand breaks induced by X-rays. Cancer Sci 2009; 100: 747-52.

[7] Kuwahara Y, Mori M, Oikawa T, Shimura T, Ohtake Y, Mori S, Ohkubo Y and Fukumoto M. The modified high-density survival assay is the useful tool to predict the effectiveness of fractionated radiation exposure. J Radiat Res 2010; 20: 297-302.

[8] Fukumoto M, Amanuma T, Kuwahara Y, Shimura T, Suzuki M, Mori S, Kumamoto H, Saito Y, Ohkubo Y, Duan Z, Sano K, Oguchi T, Kainuma K, Usami S, Kinoshita K, Lee I and Fukumoto M. Guanine nucleotide-binding protein 1 is one of the key molecules contributing to cancer cell radioresistance. Cancer Sci 2014; 105: 1351-9.

[9] Kuwahara Y, Roudkenar MH, Urushihara Y, Saito Y, Tomita K, Roushandeh AM, Sato T, Kurimasa A and Fukumoto M. Clinically relevant radioresistant cell line: a simple model to understand cancer radioresistance. Med Mol Morphol 2017; 50: 195-204.

[10] Kuwahara Y, Tomita K, Urushihara Y, Sato T, Kurimasa A and Fukumoto M. Association between radiation-induced cell death and clinically relevant radioresistance. Histochem Cell Biol 2018; 150: 649-59.

[11] Gongora C, David G, Pintard L, Tissot C, Hua TD, Dejean A and Mechti N. Molecular cloning of a new interferon-induced PML nuclear body-associated protein. J Biol Chem 1997; 272: 19457-63.

[12] Lu X, Qin B, Ma Q, Yang C, Gong XY and Chen LM. Differential expression of ISG20 in chronic hepatitis B patients and relation to interferon-alpha therapy response. J Med Virol 2013; 85: 1506-12.

[13] Dai L, Bai L, Lin Z, Qiao J, Yang L, Flemington EK, Zabaleta J and Qin Z. Transcriptomic analysis of KSHV-infected primary oral fibroblasts: the role of interferon-induced genes in the latency of oncogenic virus. Oncotarget 2016; 7: 47052-60.

[14] Lin SL, Wu SM, Chung IH, Lin YH, Chen CY, Chi HC, Lin TK, Yeh CT and Lin KH. Stimulation of interferon-stimulated gene 20 by thyroid hormone enhances angiogenesis in liver cancer. Neoplasia 2018; 20: 57-68.

[15] Van Tong H, Hoan NX, Binh MT, Quyen DT, Meyer CG, Song LH, Toan NL and Velavan TP. Interferon-stimulated gene 20 kDa protein serum levels and clinical outcome of hepatitis B virus-related liver diseases. Oncotarget 2018; 9: 27858-71.

[16] Kuwahara Y, Oikawa T, Ochiai Y, Roudkenar MH, Fukumoto M, Shimura T, Ohtake Y, Ohkubo Y, Mori S, Uchiyama Y and Fukumoto M. Enhancement of autophagy is a potential modality for tumors refractory to radiotherapy. Cell Death Dis 2011; 2: e177.

[17] Utsumi H and Elkind MM. Requirement for repair of DNA double-strand breaks by homologous recombination in split-dose recovery. Radiat Res 2001; 155: 680-6.

[18] Mattei M, Tissot C, Gongora C and Mechti N. Assignment of ISG20 encoding a new interferon-induced PML nuclear body-associated protein, to chromosome 15q26 by in situ hybridization. Cytogenet Cell Genet 1997; 79: 286-7.

[19] Zheng Z, Wang L and Pan J. Interferon-stimulated gene 20-kDa protein (ISG20) in infection and disease: review and outlook. Intractable Rare Dis Res 2017; 6: 35-40.

[20] Espert L, Rey C, Gonzalez L, Degols G, Chelbi-Alix MK, Mechti N and Gongora C. The exonuclease ISG20 is directly induced by synthetic dsRNA via NF-kappaB and IRF1 activation. Oncogene 2004; 23: 4636-40.

[21] Couté Y, Kindbeiter K, Belin S, Dieckmann R, Duret L, Bezin L, Sanchez JC and Diaz JJ. ISG20L2, a novel vertebrate nucleolar exoribonuclease involved in ribosome biogenesis. Mol Cell Proteomics 2008; 7: 546-59.

[22] Gongora C, Degols G, Espert L, Hua TD and Mechti N. A unique ISRE, in the TATA-less human Isg20 promoter, confers IRF-1-mediated responsiveness to both interferon type I and type II. Nucleic Acids Res 200; 28: 2333-41.

[23] Pentecost BT. Expression and estrogen regulation of the HEM45 mRNA in human tumor lines and in the rat uterus. J Steroid Biochem Mol Biol 1998; 64: 25-33.

[24] Rohrmus B, Thoma-Greber EM, Bogner JR and Rocken M. Outlook in oral and cutaneous Kaposi's sarcoma. Lancet 2000; 356: 2160.

[25] Gorsky M and Epstein JB. A case series of acquired immunodeficiency syndrome patients with initial neoplastic diagnoses of intraoral Kaposi's sarcoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2000; 90: 612-7.